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TITLE: ***Molecular Signatures of Chronic Pain Subtypes***

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14. ABSTRACT This project was a biomarker discovery and novel analgesic pathway discovery program focusing on the causes of persistent pain after traumatic amputation in the combat setting. During this project we have: 1) maintained the necessary regulatory approval at WRNMMC and Duke University. We have: 1) obtained and maintained documents for approval by MRMC; 2) completed patient enrollment at 124 patients; 3) maintained our interactive, secure web based data collection system; 4) populated our biorepository at Duke with bioresource collected from 124 patients enrolled at WRNMMC; 5) conducted further on-site visits and investigator meetings at WRNMMC; 6) Received and analysed all data from whole exome sequencing, gene expression, DNA methylation, miRNA, proteomic and metabolomic analysis 7) and begun follow up work on these putative pain biomarkers. We have published three papers, have submitted another and have two more in process. We have achieved four major goals: 1) Defined the clinical nature and incidence of chronic pain subtypes in traumatic military amputees 2) Demonstrated the utility of a diagnostic post-amputation pain adjudication algorithm 3) Found that regional anesthesia catheter placement is associated with reduced chronic neuropathic pain in amputees 4) 4. Discovered two novel nociceptive pathways that may serve both as biomarkers of pain and novel analgesic targets and we have received follow-up funding to further study these pathways.					
15. SUBJECT TERMS Nothing listed					
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INTRODUCTION: The Molecular Markers Of Chronic Pain Subtypes grant funding the Veterans Integrated Pain Evaluation Research (VIPER) Study was designed with two aims in mind: The first was to better categorize and describe the subtypes of chronic residual limb after amputation and the second was to identify pain biomarkers and novel analgesic pathways using a systems biology approach. After successfully enrolling 124 amputees from Walter Reed National Military Medical Center, collecting blood samples and granular phenotypic data we have successfully, and for the first time in a rigorous way, defined the clinical subtypes of chronic residual limb pain and we have identified multiple inflammatory and neural plasticity-related pathways that are differentially regulated in amputees with significant chronic pain.

KEYWORDS: *Chronic Pain, Chronic Post-surgical pain, Chronic residual limb pain, Chronic phantom limb pain, Neuropathic pain, Regional anesthesia, neuroinflammation, neural plasticity.*

OVERALL PROJECT SUMMARY: The Molecular Markers of Chronic Pain Subtypes study has successfully met all goals described in the initial application. This study was designed to better define the types of chronic pain that occur after amputation and to find molecular markers of those subtypes with the hope that this would highlight novel analgesic pathways for future treatment. In addition, we found that existing battlefield protocols encouraging far-forward regional anesthesia catheters with extended treatment duration were associated with reduced neuropathic pain after amputation. The four major results from this study are summarized below:

- Metabolomic and DNA methylation results from this trial highlighted two putative novel analgesic pathways that are now being studied by our group in a follow on grant titled “VIPER: Chronic Pain after Amputation: Inflammatory Mechanisms, Novel Analgesic Pathways, and Improved Patient Safety.” These two markers of residual limb pain, TGR5 and wnt, may offer new, non-opioid analgesic targets.
- This study demonstrated that the clinical diagnostic algorithm we used for intrastudy post-amputation pain subtype adjudication agrees well with known rates of residual limb pain incidence and highlights the incidence of more specific types of neuropathic pain (neuroma and complex regional pain syndrome (CRPS)) that have previously been poorly defined in the literature.
- This study found that the presence of regional anesthesia catheters in this patient population was associated with decreased rates of chronic neuropathic pain, highlighting the importance of continuing to encourage far-forward placement of regional catheters on the battlefield.
- Exome sequencing data revealed polymorphisms in a number of important neuroplastic and inflammatory pathways in patients who went on to develop significant residual limb pain and cytokine array data suggest that an unresolved pro-inflammatory environment may be responsible for the development of chronic post-amputation pain.

Study Task 1

We will enroll subjects between 3 and 18 months after amputation for traumatic injury in an observational study of different subtypes of post amputation chronic pain.

(a) Human subjects approval.

We expect this subtask to take 6-9 months. We will obtain IRB approval at Walter Reed Army Medical Center, in conjunction with our collaborator Dr Buckenmaier and his colleagues at DVPMI. We will submit the initial request for approval by the end of January 2011, and are advised the process is lengthy and may require several resubmissions. The first project milestone is thus IRB approval to enroll subjects at WRAMC, and we expect to reach this no later than 10/1/2011.

(b) Human subject enrollment.

We expect to enroll 165 amputee soldiers at WRAMC over the course of this 3-year project. The second project milestone is enrollment of the first subject by 12/1/2011. In order for the proteomic experiments to have sufficient power we need a minimum of 90 subjects. We will try and enroll as many subjects as we can in the first 12 months after IRB approval. There are several hundred potentially eligible patients undergoing treatment at MATC as of December 2010. The third project milestone is thus enrollment of a minimum of 90 subjects by 12/1/2012.

Study Task 1 – Year 4 Summary Report

We completed enrollment of 124 patients. We maintained a near 100% completion of the case report forms, with less than 1% missing data. In brief, there are 124 patients of whom 80 (64%) are cases and 44 (36%) controls. The incidence of residual limb pain is 76/124 (61%) and of phantom pain 76/124 (61%). The subtypes of residual limb pain are neuroma (37/124), mosaic (8/124), CRPS (15/124) and somatic (31/124). While these incidences are in keeping with the previously published rates, the pain subtyping process is a novel contribution to the scientific literature, and is summarized in Table 1 below.

Table 1: Chronic pain subtypes following adjudication of amputees enrolled in the Veterans Integrated Pain Evaluation Research Study

Pain Phenotypes			
Total Patients Enrolled	All Patients n=124		
Any Patient with Pain (NRS>0)	Any Pain n=115 (92.7%)		
Patients with Significant Pain (NRS≥3)	All Significant Pain n=80 (64.5%)		
Residual Limb Pain (NRS≥3)	Significant Residual Limb Pain n=76 (61%)		
Phantom Limb Pain (NRS≥3)		Significant Phantom Limb Pain n=72 (58%)	
Phenotypic Subtypes of Significant Residual Limb Pain			
Neuroma (n=37, 48.7% of RLP)	2	+35 Neuroma	
Somatic (n=31, 40.8% of RLP)	4		+27 Somatic
CRPS (n=15, 19.7% of RLP)		CRPS n=15	
Mosaic (n=8, 10.5% of RLP)	2		+6 Mos

Study Task 2 – Biomarker Discovery (Aims 1 & 2)

i. Proteomics

Duration 12 months

Milestone Final data back from Duke Core

ii. Genotyping

Duration 6 months

Milestone Final data back from Duke Core

iii. Sequencing

Duration 9 months

Milestone Final data back from Duke Core

Study Task 2 – Year 3 Summary Report

Proteomic and Metabolomic Discovery Subtask (i)

We paralleled our human biomarker and novel pathway discovery work in humans with a mouse peripheral nerve injury model that was developed under the direction of another member of our lab, Dr. Thomas Van de Ven. This project used a mouse peripheral nerve injury model that approximates the pathology present in human amputees. We have performed metabolomic analysis of various mouse tissues from this model, including blood plasma, for cross-species verification of potential biomarkers of interest.

We completed metabolomic analysis on the 80 patient VIPER discovery cohort and used the resulting data, with cross-species verification in mice, to identify a bile acid signaling pathway as differentially expressed in patients with pain after amputation. This pathway, which includes the g-protein coupled receptor TGR5 and the nuclear receptor FXR, is known to be a signaling pathway important in metabolism and inflammation but only recently has there been evidence that it may play a role in nociception. We used the results from our mouse model and VIPER

patient cohort to argue that TGR5 may have a role as a novel analgesic target and we have begun to study this hypothesis using funding from a follow-on CDMRP Neurosensory grant.

Table 2: Ratio of metabolite concentration between case and control in mice and humans. In mice, cases have spared nerve injury and controls have sham surgery. In humans, cases have pain after amputation and controls do not. Green shading shows increase in metabolite concentration in cases vs controls and red shows decrease concentration in cases vs. controls.

Species	Sub pathway	Metabolite	SNI/sham	p-value
Mouse	Bile acid metabolism	cholate	0.44	0.0437
Mouse		deoxycholate	0.23	2.59E-05
Mouse		beta-muricholate	0.26	0.0006
Mouse		taurodeoxycholate	0.37	0.0124
Human	Bile acid metabolism	cholate	0.59	0.0677
Human		deoxycholate	1.79	0.0392
Human		taurodeoxycholate	0.54	0.0525

We also completed unbiased proteomic analysis of blood plasma from the 80 patient VIPER discovery cohort and found a number of differentially expressed proteins listed in Table 3, including a number involved in neuroinflammation.

Table 3: Differentially expressed proteins as a function of pain identified from blood plasma collected 3-18 months after amputation.

Primary Protein Name	Protein Description	Unique Peptides	ProteinTeller Probability	Fold-Change (Case vs Control)	ANOVA P-value
FHR5_HUMAN	Complement factor H-related protein 5 OS=Homo sapiens GN=CFHR5 PE=1 SV=1	3	1.0	-1.12	0.009
FIBA_HUMAN	Fibrinogen alpha chain OS=Homo sapiens GN=FGA PE=1 SV=2	32	1.0	1.15	0.011
C1S_HUMAN	Complement C1s subcomponent OS=Homo sapiens GN=C1S PE=1 SV=1	19	1.0	1.06	0.013
FA10_HUMAN	Coagulation factor X OS=Homo sapiens GN=F10 PE=1 SV=2	7	1.0	1.05	0.022
CYTC_HUMAN	Cystatin-C OS=Homo sapiens GN=CST3 PE=1 SV=1	2	1.0	1.19	0.024
ENO4_HUMAN	Enolase-like protein ENO4 OS=Homo sapiens GN=ENO4 PE=2 SV=2	1	0.9	-1.10	0.040
F13B_HUMAN	Coagulation factor XIII B chain OS=Homo sapiens GN=F13B PE=1 SV=3	7	1.0	1.08	0.043
C1R_HUMAN	Complement C1r subcomponent OS=Homo sapiens GN=C1R PE=1 SV=2	17	1.0	1.05	0.045

We plan to perform a similar experiment in our mouse model to provide cross-species verification of these results in the near future.

We also performed cytokine array analysis on the 80 patient VIPER discovery cohort and found that a number of pro-inflammatory cytokines were upregulated in these patients. This is surprising since these patients should be fully recovered from initial injury with any accompanying inflammatory response already resolved. Our findings suggest that the inflammatory processes generated by amputation may last much longer than previously thought and may be responsible for the deleterious transition from acute to chronic post-amputation pain. These results are summarized in Table 4 below and have been submitted as a manuscript to the journal Pain. That manuscript is now in the revision stage and likely to be published in the next few months.

Table 4. Inflammatory mediators found to either positively or negatively correlate with average residual limb pain intensity and pain catastrophizing using blood plasma from patients 3-18 months after amputation.

Systemic Mediator	Average Pain	P value	PCS	P value
IL-13	-0.45	0.000	-0.29	0.010
IL-8	0.26	0.024	0.25	0.030
IL-12	0.31	0.006	0.24	0.037
TNF- β	0.43	0.000	0.39	0.001
PIGF	0.31	0.008	0.34	0.003
Tie2	0.35	0.002	0.22	0.059
ICAM-1	0.43	0.000	0.44	0.000

Genotyping and Epigenetic Discovery Subtask (ii)

We completed DNA methylation array analysis on the 80 patient VIPER discovery cohort. This is an unprecedented dataset and represents an incredible opportunity to learn the mechanisms underlying the transition from acute to chronic pain in military amputees. We are currently working with our statistician, Dr. Yi-Ju Li, who is an Associate Professor in the Department of Biostatistics and Bioinformatics at the Center for Human Genetics in the Duke Department of Medicine. She and her postdoctoral candidate are performing the complex convergent pathway analysis required to complete our search for novel pain biomarkers and pathways. The initial results led to identification of the mapk and wnt pathways as differentially methylated in patients with and without pain after amputation. The MAPK pathway has long been studied as an important regulator of nociception, but wnt has only recently been recognized as an important generator of nociceptor sensitivity. Because of these findings, we have received follow on funding to study the role of the wnt pathway in neuropathic pain. Over the next three years we hope to continue targeted validation of these findings in both this VIPER cohort and in the VIPER Valproate cohort being collecting in a separate study lead by a co-investigator on this grant, Dr. Thomas Buchheit. Also, we hope to determine whether the wnt pathway alters nociception due to direct effects on neuroplasticity, modulation of neuroinflammation, or both. Table 5, below, lists the wnt signaling pathway members would to be differentially methylated in VIPER patients with residual limb pain compared to those without.

Table 5: Wnt pathway constituents (from DAVID online functional annotation tool) with specific CpG locations hypomethylated in VIPER patient cases vs controls.

Gene	CpG site	Delta beta	P-value	FDR
CREBBP	cg04336433	-0.1	0.003223791	0.055758138
TBL1X	cg04414946	-0.1	0.00167226	0.055758138
NFATC1	cg07740306	-0.1	0.002908787	0.055758138
PRKCG	cg14045992	-0.1	0.003243531	0.055758138
CAMK2B	cg09126559	-0.1	7.59977E-04	0.055758138
<i>Wnt5a</i>	cg17553300	-0.1	3.61E-06	0.047041308
CTBP1	cg07825433	-0.11	0.002410452	0.055758138
NFATC1	cg21848624	-0.11	0.005915706	0.058800131
CAMK2B	cg06746426	-0.12	0.001388434	0.055758138
PRICKLE2	cg03170262	-0.12	0.002224953	0.055758138
NKD2	cg12192884	-0.12	0.00440416	0.056502306
CTBP2	cg15205441	-0.12	0.001907882	0.055758138
LRP5	cg07985116	-0.15	0.003865465	0.05588898

CREBBP	cg01383349	-0.16	9.67033E-04	0.055758138
CCND1	cg06741896	-0.17	0.003372107	0.055758138
SMAD3	cg07882838	-0.2	8.21618E-04	0.055758138

Whole Exome Sequencing Subtask (iii)

We also successfully performed whole exome sequencing on the entire 124 patient cohort and received all data back from the sequencing core facility. We completed full analysis of this dataset. As planned, this cohort did not have the power to identify individual polymorphisms that may be associated with pain, but we were able to identify multiple neuroinflammatory and neuroplasticity related pathways containing polymorphisms in amputees with residual limb pain. Using Ingenuity IPA software, we were able to obtain a list of most enriched pathways. These pathways are found below in Table 6. The manuscript describing these findings is now in preparation. Interestingly, the most significantly enriched pathway, STAT3, has recently been implicated as very important in the pathogenesis of chronic neuropathic pain. Also, the FXR/RXR pathway is a bile acid nuclear receptor pathway and provides cross-molecular confirmation of our metabolomics based finding that the bile acid pathway is important in chronic post-amputation pain.

Table 6: Pathways found to be enriched in rare variants when comparing whole exome sequencing results in amputees with significant chronic post-amputation pain and those without. Results from Ingenuity IPA core analysis.

Ingenuity Canonical Pathways	-log(p-value)	Ratio
STAT3 Pathway	3.14E00	1.64E-01
LXR/RXR Activation	2.91E00	1.32E-01
Nitric Oxide Signaling in the Cardiovascular System	2.86E00	1.4E-01
NF-κB Signaling	2.75E00	1.16E-01
FXR/RXR Activation	2.69E00	1.26E-01
eNOS Signaling	2.58E00	1.2E-01
Neuropathic Pain Signaling In Dorsal Horn Neurons	2.41E00	1.3E-01
Virus Entry via Endocytic Pathways	2.39E00	1.35E-01
nNOS Signaling in Neurons	2.36E00	1.7E-01
Caveolar-mediated Endocytosis Signaling	2.21E00	1.41E-01
PTEN Signaling	2.19E00	1.19E-01
Prolactin Signaling	2.13E00	1.37E-01
Axonal Guidance Signaling	2.12E00	8.55E-02
Assembly of RNA Polymerase I Complex	1.94E00	3.33E-01
IL-15 Production	1.8E00	1.85E-01
T Helper Cell Differentiation	1.75E00	1.27E-01
IL-12 Signaling and Production in Macrophages	1.7E00	1.04E-01
NF-κB Activation by Viruses	1.68E00	1.23E-01
Aldosterone Signaling in Epithelial Cells	1.62E00	9.87E-02
Neuregulin Signaling	1.59E00	1.14E-01
VEGF Family Ligand-Receptor Interactions	1.58E00	1.18E-01
IL-4 Signaling	1.58E00	1.18E-01
IL-17A Signaling in Airway Cells	1.58E00	1.25E-01
Tyrosine Degradation I	1.55E00	4E-01
Gap Junction Signaling	1.55E00	9.68E-02
Non-Small Cell Lung Cancer Signaling	1.54E00	1.23E-01

Synaptic Long Term Potentiation	1.45E00	1.01E-01
TCA Cycle II (Eukaryotic)	1.43E00	1.74E-01
Aggrin Interactions at Neuromuscular Junction	1.41E00	1.16E-01
Growth Hormone Signaling	1.41E00	1.16E-01
Glutamate Receptor Signaling	1.4E00	1.23E-01
Glioma Signaling	1.39E00	1.05E-01
tRNA Splicing	1.35E00	1.43E-01

KEY RESEARCH ACCOMPLISHMENTS

- Completed patient enrollment at 124.
- Completed genome wide DNA methylation, miRNA array, unbiased plasma proteomic and global plasma metabolomic data received on 79 patient discovery cohort and whole exome sequencing on all 124 patients
- Multiple putative pain pathways identified with two pathways chosen for directed mechanistic investigation. Each of these pathways (TGR5/FXR and Wnt) may provide potential therapeutic targets in the future. This follow on work is being funded by Department of Defense CDMRP grant W81XWH-15-2-0046.
- Omega-3 fatty acid levels from VIPER metabolomics correlated with post-amputation pain leading to testing of omega-3 fatty acid supplementation in mice with nerve injury and publication submitted.
- Cytokine array analysis revealed significant differences in multiple cytokines between amputees with and without pain. These results have been submitted for publication in the journal Pain and that manuscript is now in revision.
- Follow-on intervention study currently enrolling (PT110575, T Buchheit PI).
- Follow-on CDMRP Neurosensory Research Award proposal (MR130082) funded to study the analgesic potential of the pathways discovered from VIPER data analysis.
- Two original research publications completed, four review articles completed, one original research publication in revision, and one original research article actively in preparation.
- 10 conference abstracts and posters presented at regional and national pain and anesthesiology meetings

CONCLUSION: The clinical and biological data collected in this study have not only helped define the incidence and nature of pain subtypes after amputation but have revealed a number of signaling pathways that may serve both as biomarkers of chronic post-amputation pain but have provided the insight necessary to continue study of these pathways with the goal of novel non-opioid analgesic development. In addition, this study showed a significant association between the presence of regional anesthesia catheters and reduced incidence of neuropathic pain after amputation suggesting that the current military efforts to provide far-forward regional anesthesia may be reducing the burden of chronic post-nerve injury pain in battlefield amputees.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Publications:

1. Zillioux J, Chameessian A, Mauck M, Buchheit T, Van de Ven T, “Oral supplementation with the omega-3 fatty acids docosahexaenoic (DHA) and eicosapentaenoic (EPA) attenuates

mechanical allodynia in a mouse model of peripheral nerve injury.” *Matters*. Epub February 2016.

2. Alexander Chameessian, Thomas Van de Ven (co-first author), Thomas Buchheit, Hung-Lun Hsia, Mary McDuffie, Eric Gamazon, Colin Walsh, Stephen Bruehl, Chester ‘Trip’ Buckenmaier III, Andrew Shaw. Differential Expression of Systemic Inflammatory Mediators in Amputees with Chronic Residual Limb Pain.” In revision, *Pain*, 2016.
3. Buchheit T, Van de Ven T, John Hsia HL, McDuffie M, MacLeod DB, White W, Chameessian A, Keefe FJ, Buckenmaier CT, Shaw AD. “Pain Phenotypes and Associated Clinical Risk Factors Following Traumatic Amputation: Results from Veterans Integrated Pain Evaluation Research (VIPER).” *Pain Med*. 2015 Jul 14. doi: 10.1111/pme.12848. [Epub ahead of print]
4. Mauck, M, Van de Ven T, Shaw, A. “Epigenetics of Chronic Pain after Thoracic Surgery.” *Current Opinion in Anesthesiology*. 2014, 27:1–5.
5. Buchheit T, Van de Ven T, Shaw AD. “Epigenetics and the transition from acute to chronic pain.” *Pain Med*. 2012 Nov;13(11):1474-90.
6. Van de Ven T, Hsia HL. “Causes and Prevention of Chronic Postsurgical Pain.” *Current Opinions in Critical Care*. 18(4):366-71 August 2012.
7. Buchheit, T, and Pyati, S. "Prevention of chronic pain after surgical nerve injury: amputation and thoracotomy." *Surg Clin North Am*. 2012 Apr;92(2):393-407

Abstracts:

Abstract submitted and posters presented at the American Society of Anesthesiologists (ASA) Conference 2012

Pre-Operative Dexamethasone Decreases the Development of Chronic Mechanical Allodynia in a Mouse Tibial Spared Nerve Injury Model.

Sub-Anesthetic Ketamine Prior to Nerve Lesion Reduces the Development of Chronic Neuropathic Pain in a Mouse Tibial Spared Nerve Injury Model.

Pain Candidate Pathway Priorization Using Interspecies Plasma Metabolomics.

Veterans Integrated Pain Evaluation Research (VIPER): Post-amputation Pain Phenotypes in Injured Military Service Personnel.

Veterans Integrated pain Evaluation Research (VIPER) Pilot Cohort: Feasibility of Studying Combat Amputation Pain.

Posters presented at ASA Conference 2013

Genome wide DNA methylation analysis in amputees with chronic residual limb pain reveals significant epigenetic regulation of the MAPK pathway.

Regional Anesthesia Catheters Reduce the Incidence of Chronic Neuropathic Pain After Traumatic Amputation: Initial Results From the VIPER-80 Discovery Cohort of Injured Military Personnel.

Oral presentation for “10 Best Abstracts” symposium at ASA Conference 2013

Whole Exome Sequencing Identifies Novel Genetic Variants in Amputees With Persistent Residual Limb Pain.

Poster presented at PSOC Conference 2013

More than Mere Detergents: An Interspecies Study Reveals Bile Acids as Novel Mediators in Acute and Chronic Pain

Abstract submitted and poster presented at PSOC conference 2014

Inflammatory Biomarkers in Patients with Persistent Post-operative Pain after Amputation

Abstract submitted and poster presented at AAPM Conference 2014

Regional Anesthesia Catheters Reduce the Severity of Neuropathic Post-Amputation Pain: Initial Results from the VIPER-80 Discovery Cohort of Injured Military Personnel

INVENTIONS, PATENTS AND LICENSES:

N/A

REPORTABLE OUTCOMES:

N/A

OTHER ACHIEVEMENTS:

Research Opportunity Applied for and Received

Follow-on DMRDP Neurosensory Research Award (MR130082) to study the analgesic potential of the pathways discovered from VIPER data analysis received in 2015. CDMRP W81XWH-15-2-0046: VIPER: Chronic Pain after Amputation: Inflammatory Mechanisms, Novel Analgesic Pathways, and Improved Patient Safety

Dr. Thomas Van de Ven, collaborator on this project, received an intramural DREAM Innovation Grant to study the TGR5/FXR pathway discovered using VIPER metabolomic data in 2014.

Dr. Thomas Buchheit, Co-Investigator on this project, submitted a proposal in response to the Department of Defense Program Announcement, Psychological Health/Traumatic Brain Injury Research Program, Funding Opportunity Number W81XWH-11-PHTBI-ANRA, submitted on January 6, 2012. He received Notice of Award and his project titled “Regional Anesthesia and

Valproate Sodium for the Prevention of Chronic Post-Amputation Pain” started September 30, 2012, a four-year project.

REFERENCES:

N/A

APPENDICES:

Quad Chart

Abstracts

Publications

Molecular Signatures of Chronic Pain Subtypes

Log# DM102142

Award Number W81XWH-11-2-0003



PI: Andrew D Shaw MD

Org: Duke University

Award Amount: \$1,336,109.00

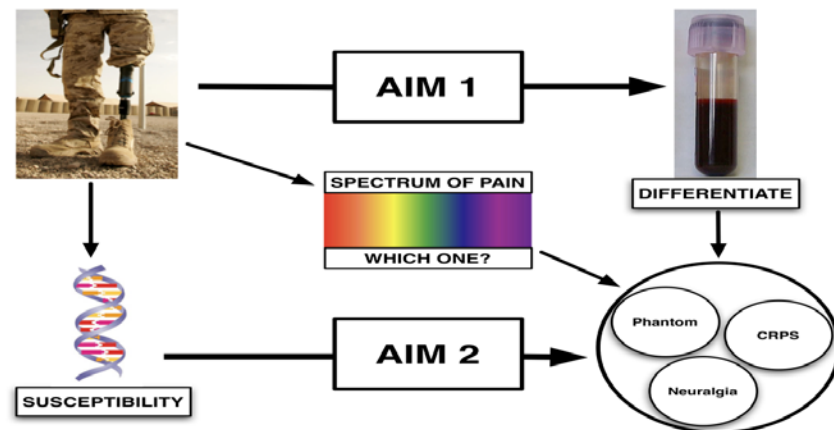
Study/Product Aim(s)

• **Problem:** (1) There are no good tests to differentiate between different types of chronic amputation pain. (2) There are no good ways to measure susceptibility to chronic pain subtypes (phantom limb pain, neuralgia, CRPS).

• **Hypothesis:** (1) Convergent pathway analysis from multiple data types including exome sequence, DNA methylation, gene expression, proteomics and metabolomics will reveal biomarkers of susceptibility to pain type.

Approach

- 1) Describe the molecular signatures of phantom pain, neuralgia pain and Complex Regional Pain Syndrome (CRPS) in 124 war injured amputees.
- 2) Describe the biology of chronic amputation pain subtypes in terms of the proteins and metabolites expressed in blood samples from amputees.
- 3) Generate a list of candidate risk genes for each subtype of amputation pain.
- 4) Sequence genes to find new polymorphisms associated with functional differences and risk of each pain subtype.



Timeline and Cost

Activities	CY	11	12	13	14
Patient enrollment					
Circulating biomarker discovery					
Gene sequencing					
Validation Studies					
Estimated Budget (\$K)		\$481	\$449	\$406	NCE

Goals/Milestones

CY11 Milestones-

- ✓ All protocol documents submitted and approved 12/23/11

CY12 Milestones-

- ✓ Patient enrollment began
- ✓ Received funding to reduce the incidence of chronic post-amputation pain. PT110575 (PI: Buchheit)

CY13 Milestones-

- ✓ Completed patient enrollment
- ✓ Received funding to further study pathways and biomarkers of interest found from this dataset. MR130082 (PI: Van de Ven)

CY14/15 Milestones –

- ✓ All unbiased 'omic datasets collected.
- ✓ Publication of clinical data, cytokine array data
- ✓ Preparation of exome sequencing and methylation results manuscripts
- ✓ Publication of manuscript describing the correlation between pain and plasma omega-3 fatty acid levels.

Budget Expenditure to Date (as of 12 December 2014)

Projected Expenditure: \$1,336,109.00

Actual Expenditure: \$1,336,100.48

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Title: Perioperative supplementation with omega-3 fatty acids may attenuate post-surgical neuropathic pain.

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ABSTRACT

Objective: The omega-3 fatty acids docosahexaenoic (DHA) and eicosapentaenoic (EPA) are precursors to a family of analgesic and neuroprotective small pro-resolution lipid mediators (PRLMs) that include the resolvins and neuroprotectins. We hypothesized that perioperative supplementation with DHA and EPA can prevent post-surgical pain by increasing endogenous levels of PRLMs.

Methods: To identify targets for novel analgesics, our lab conducted a global metabolomics study of 80 human patients with traumatic amputations as part of the Veterans Integrative Pain Evaluation Research clinical trial. We analyzed the results of this study for associations between omega-3 fatty acids and pain severity. We then treated nerve-injured mice with perioperative oral DHA and EPA with or without aspirin to determine whether DHA, EPA and their PRLM metabolites reduce mechanical allodynia in a mouse model of peripheral nerve injury.

Results: There was a negative correlation between DHA and EPA concentration and neuropathic pain severity in human traumatic amputees. We found that mice treated with both DHA/EPA or DHA/EPA with aspirin had significantly reduced mechanical allodynia in the ipsilateral paw compared to injured control animals. There was no significant difference in allodynia reduction between the treatment groups. Also, there was a trend toward increased plasma PRLMs neuroprotectin D1 and protection DX in mice treated with DHA and EPA with aspirin.

Conclusion: Our results suggest that perioperative DHA/EPA supplementation may provide a safe, inexpensive and effective way to increase PRLM levels and prevent chronic pain in humans.

Keywords: Chronic postoperative pain, inflammation, neuropathic pain, omega-3 fatty acids

INTRODUCTION

Surgical procedures, including mastectomy, amputation, and thoracotomy, are followed by severe and disabling chronic neuropathic pain in 5-10% of cases (1). This post-surgical neuropathic pain and other chronic pain syndromes represent an enormous public health burden, annually costing the United States upwards of \$630 billion in direct health expenses and lost productivity (2). Despite our success at managing acute post-surgical pain, current therapeutic options for the chronic pain that follows are limited. As such, there is an immediate need for novel preventive analgesics.

Chronic neuropathic pain is believed to partially result from neuroinflammation following nerve injury and subsequent peripheral and central sensitization that occurs as a consequence of this inflammation (1-3). As it has become increasingly appreciated that initiation and resolution of inflammation are distinct processes (4), novel therapeutic strategies aimed at interrupting the inflammation and switching to a resolving state seem especially promising.

Growing evidence suggests that the novel pro-resolving lipid mediator (PRLM) metabolites of the omega-3 fatty acids docosahexaenoic (DHA) and eicosapentaenoic acids (EPA) may achieve this aim. These oxylipins, which include resolvins, neuroprotectins, and maresins, are generated via several metabolic pathways involving lipoxygenase (LOX) or cyclooxygenase (COX) enzymes at sites of tissue injury (5,6). Of note, there are two R-series resolvins that are generated from DHA and EPA by the

aspirin acetylated COX2 enzyme (5). Pro-resolving oxylipins have proven potent anti-inflammatory agents: for example, resolvins are 1000 times more effective than DHA or EPA and 100 times more effective than morphine at mitigating inflammatory pain (7). Resolvins and neuroprotectins have been extensively studied in multiple rodent models of pain and found to prevent as well as treat established inflammatory, post-surgical, and neuropathic pain (6,10). These effects are attributed to their ability to actively resolve inflammation as well as inhibit neural plasticity, glial activation, and transient receptor potential (TRP) channels (8-10).

The pro-resolving properties of resolvins and neuroprotectins likely explain the wide therapeutic applications of omega-3 polyunsaturated fatty acids (PUFAs). Therapeutic benefit of supplementation with omega-3 PUFAs has long been suspected in inflammatory and cognitive diseases (11,12), and has been well established in the case of heart disease despite incomplete understanding of its underlying mechanisms (13,14). By providing the raw material to enhance PRLM production at the site of injury, omega-3 fatty acid supplementation may act as a safe and novel preventive analgesic therapy.

We have recently completed enrollment of one hundred and twenty four recent active duty military post-traumatic amputees at Walter Reed National Military Medical Center receiving care at the Defense and Veterans Center for Integrative Pain Medicine (DVCIPM) under the Veterans Integrated Pain Evaluation Research Study (VIPER). Patients were enrolled three to eighteen months after amputation. Each patient was categorized as “case” or “control” based on S-LANSS severity score and blood samples were drawn for multiple types of data analysis, including global metabolomics profiling

that included quantification of plasma fatty acids allowing correlation with post-amputation pain scores.

In addition, we use a murine spared-nerve injury model to test whether perioperative supplementation with omega-3 PUFAs: (A) Increases endogenous levels of pro-resolving oxylipins in mice with co-existing peripheral nerve injury, (B) attenuates chronic neuropathic pain in a mouse model of peripheral nerve injury and (C) if the addition of aspirin augments pro-resolving oxylipin levels and improves pain relief.

METHODS

VIPER Study Design and sample collection

After IRB approval, 124 subjects were enrolled at Walter Reed National Military Medical Center (WRNMMC) in this retrospective cohort study. Multiple pain and psychometric questionnaires were administered to each individual to be completed with minimal guidance by a healthcare provider. One of the included questionnaires was the self-report version of the Leeds Assessment of Neuropathic Symptoms and Signs score (S-LANSS), which includes a question asking each patient to report average pain severity in the affected limb over the past week. We termed this question the S-LANSS severity score. Patients were designated as “cases” if they had an S-LANSS severity score greater or equal to 3.

Subjects were included if they were a military health care beneficiary age 18 years or older and undergoing treatment at WRNMMC with a diagnosis of post injury amputation of all or part of one limb. Amputation injury must also have occurred between 3 and 18 months prior to enrollment.

Patients were excluded if they were afflicted with severe traumatic brain injury, significant cognitive deficits, substantial hearing loss, spinal cord injury with permanent or persistent deficits, ongoing tissue damage pain, infection, bone spur, poorly fitting prosthesis, or hip disarticulation. Blood samples were collected in EDTA containing tubes, centrifuged for 15 minutes at 1600G at 4°C within ~30 minutes of sample collection, and plasma pipetted off and aliquoted into 1.8ml cryovials.

Unbiased Plasma Metabolomics

Metabolomics experiments were conducted by Metabolon Inc (Durham NC)

Sample Preparation: The sample preparation process was carried out using the automated MicroLab STAR® system from Hamilton Company. Recovery standards were added prior to the first step in the extraction process for QC purposes. Sample preparation was conducted using a proprietary series of organic and aqueous extractions to remove the protein fraction while allowing maximum recovery of small molecules. The resulting extract was divided into two fractions: one for analysis by LC and one for analysis by GC. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. Each sample was then frozen and dried under vacuum. Samples were then prepared for the appropriate instrument, either LC/MS or GC/MS. Essential fatty acid sub-analysis required the LC/MS platform.

Liquid chromatography/Mass Spectrometry (LC/MS, LC/MS²): The LC/MS portion of the platform was based on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ mass spectrometer, which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The sample extract was split into two aliquots,

dried, then reconstituted in acidic or basic LC-compatible solvents, each of which contained 11 or more injection standards at fixed concentrations. One aliquot was analyzed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions in two independent injections using separate dedicated columns. Extracts reconstituted in acidic conditions were gradient eluted using water and methanol both containing 0.1% Formic acid, while the basic extracts, which also used water/methanol, contained 6.5mM Ammonium Bicarbonate. The MS analysis alternated between MS and data-dependent MS² scans using dynamic exclusion.

Accurate Mass Determination and MS/MS fragmentation (LC/MS), (LC/MS/MS): The LC/MS portion of the platform was based on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ-FT mass spectrometer, which had a linear ion-trap (LIT) front end and a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer backend. For ions with counts greater than 2 million, an accurate mass measurement could be performed. Accurate mass measurements could be made on the parent ion as well as fragments. The typical mass error was less than 5 ppm. Ions with less than two million counts require a greater amount of effort to characterize. Fragmentation spectra (MS/MS) were typically generated in data dependent manner, but if necessary, targeted MS/MS could be employed, such as in the case of lower level signals.

The human plasma dataset comprised a total of 363 named biochemicals and 295 unnamed compounds. Following log transformation and imputation with minimum observed values for each compound, a One Way ANOVA with Contrasts and a Welch's

t-Test were used to identify biochemicals that differed significantly between experimental groups

Animals

All animal experiments were approved by the Institutional Animal Care & Use Committee at Duke University and were conducted in accordance with the U.S. Government Principles for Utilization and Care of Vertebrate Animals for Testing, Research, and Training. 8-10 week-old male C57BL/6 mice ordered from Charles River Laboratories (Wilmington, MA) were used.

The mice were housed in cages of 5 and had access to chow and water ad libitum. The chow was either Picolab® Rodent Diet 20 (5053) or Rodent Laboratory Diet (5001) from LabDiet® (St. Louis, MO), both of which contain omega-3 to omega-6 ratios of about 0.15.

Drugs and Drug Administration

Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) were purchased from Cayman Chemical as solutions in ethanol. For these experiments, after evaporating the ethanol solvent under a gentle nitrogen stream, DHA and EPA were delivered in a soybean oil control vehicle (Crisco Pure Vegetable Oil) containing the anti-oxidant Vitamin E. Soybean oil was chosen as the control vehicle for its low omega-3 to omega-6 PUFA ratio of 0.14 (15), which is similar to the mice's background diet. Acetylsalicylic acid (ASA) was purchased from Sigma.

All treatments were delivered daily to the mice via oral gavage, on the day before surgery through post-operative day 12. Mice in the appropriate treatment groups received approximately 200 mg/kg of DHA/EPA and 30 mg/kg of ASA per treatment. Dosing estimates are based on a 26g mouse, the average mass of an adult C57BL/6 mouse. Control groups received soybean oil over the same time span. The DHA and EPA were delivered together in the soybean oil carrier. ASA was delivered in water.

Spared-Tibial Nerve Injury Surgery

Spared-tibial nerve injury (SNI) surgery was performed under 2-3% isoflurane anesthesia, as described by Shields (16). The left hind limb was immobilized in a lateral position. After skin incision at the mid-thigh level and dissection through the underlying muscle, the sciatic nerve trifurcation was exposed. The common peroneal and sural nerve branches were tightly ligated with 6-0 silk sutures and then severed. Throughout the procedure, the tibial nerve was preserved by carefully avoiding any stretch or nerve contact. For sham surgeries, the sciatic nerve trifurcation was exposed without imposing any nerve injury.

Whole blood was collected from anesthetized mice (3-4% isoflurane) via cardiac puncture. To puncture the heart, a 23-gauge needle was inserted left of and under the sternum in the direction of the head at a 25 degree angle. Once blood was seen in the syringe, negative pressure was applied to the syringe. The whole blood was collected in a 1-mL EDTA tube and centrifuged at 5000 rpm for 5 minutes at room temperature. Plasma was then collected from the top of the centrifuged tube and stored at -80C.

Behavioral Assays

Mice were habituated to the testing environment for 2 days prior to baseline testing and for at least 30 minutes on each subsequent test day. The mice were placed in plastic boxes on an elevated wire-mesh apparatus. Mechanical allodynia was assessed by stimulating the left hind paw with Von Frey filaments of logarithmically increasing stiffness (0.04 – 2.00 g, Stoelting Co, Wood Dale, IL), applied perpendicularly to the plantar surface. Specifically, the hindpaw was stimulated in the distribution of the tibial nerve, in the center of the plantar surface (17). The 50% paw withdrawal thresholds were determined using the up-down method of Dixon (18). Testing was performed by a blinded researcher at baseline, post-operative day (POD) 3 and every following third day, finishing on POD 21.

Plasma Oxylipin Assays

Plasma oxylipin assays were performed at the Duke Proteomics and Metabolomics Shared Resource. Stable Isotope Labeled (SIL) oxylipin standard solutions were purchased from Cayman Chemical (Ann Arbor, MI). Solutions were combined in a stock SIL mixture in methanol which was further diluted with acetonitrile to a final concentration of 6.25 nM (IS Working Solution). Analytical standard solutions were purchased from Cayman Chemical (Ann Arbor, MI). Solutions were combined in a stock mixture containing 1 µg of each compound in methanol which was further diluted with 1:1 acetonitrile:methanol to prepare Spiking Solutions from which Quality Control samples (QC) and calibration standards were made. Calibration standards and QCs were prepared in 50 mg/mL Bovine Serum Albumin (BSA) in 50 mM ammonium

bicarbonate (AmBic). Calibration standards were analyzed in duplicate bracketing the study samples and QCs. The concentrations of the calibration standards were 10, 25, 50, 100, 250, 500, 1000, 5000, 10000, and 50000 pg/mL. QC samples were prepared at three concentrations 40000, 4000, and 400 pg/mL. These were analyzed in duplicate with the study samples.

Samples were extracted by protein precipitation with acetonitrile using a Biotage (Uppsala, Sweden) PLD+ protein and phospholipid removal 96-well plate. Plasma samples were thawed, mixed, and spun at a slow speed to pellet any solids. For each blank, calibration standard, QC, and study sample 800 μ L IS Working Solution were added to the appropriate well of the protein and phospholipid removal 96-well plate. 800 μ L acetonitrile were added to each well to be used for double blanks. Aliquots of 90 μ L blank, calibration standard, and QC sample were added to the appropriate wells. Plasma study samples were added in 90 μ L aliquots when possible. The extraction plate was then capped, mixed for 10 minutes at room temperature, and frozen for 10 minutes at -20°C. The collection 96-well plate, containing a solution with 5 μ L glycerol as carrier, was positioned below the extraction plate in a vacuum block, then vacuum was applied for 5 minutes to elute the samples. The collected samples were dried under a gentle stream of nitrogen then reconstituted in 50 μ L 1:1 acetonitrile:methanol. 5 μ L were injected for LC/MS/MS analysis.

LC-MS/MS analysis of oxylipin molecules was performed based on the method of Laiakis et al [site <http://www.ncbi.nlm.nih.gov/pubmed/25126707>]. Briefly, UPLC separation was performed using a Waters (Milford, MA) Acquity UPLC using an Acquity 2.1 mm x 10 mm 1.7 μ m BEH C18 column. Mobile phase A was water with 0.1% acetic

acid and mobile phase B was 90:10 acetonitrile:isopropyl alcohol. Samples were introduced directly into a Xevo TQ-S mass spectrometer (Waters) using negative electrospray ionization operating in the Multiple Reaction Monitoring (MRM) mode. MRM transitions (compound-specific precursor to product ion transitions) for each analyte and internal standard were collected over the appropriate retention time. The MRM data were imported into Waters application TargetLynx™ for peak integration, calibration, and concentration calculations. Analytes for which analytical standards were not included were quantified against the standard curve of an analyte from the same or similar compound class.

Statistical Analysis

Essential fatty acids concentrations from the metabolomics dataset were chosen for further analysis using GraphPad Prism 6 software (GraphPad Software, San Diego). Linear regression analyses were performed to assess the relationship between S-LANSS severity score and the plasma levels of omega-3 PUFA and omega-6 PUFAs. Patients with S-LANSS severity scores of 9 and 10 were excluded from this analysis, as there was only 1 patient per group.

Statistical analysis of mouse behavioral data was also performed in GraphPad. Paw withdrawal thresholds were normalized to baseline and are presented as mean percent of baseline with standard error of the mean. Missing data was imputed with the mean of the shared treatment group's paw withdrawal threshold for the time point. The paw withdrawal thresholds of the treatment groups were compared using repeated-measures two-way ANOVA and ad-hoc Tukey tests corrected for multiple comparisons.

Area under the curve and quadratic polynomial regression analyses of the paw withdrawal thresholds were also performed, the details of which are available in the Supplementary Methods.

Oxylipin assay results were also analyzed in GraphPad. One outlier was excluded from each of the SNI control and DHA/EPA treatment groups. Values below the lower limit of quantification (LLQ) were imputed with the LLQ, thereby biasing towards the null hypothesis. We pre-specified comparisons of the DHA/EPA groups with and without aspirin with the SNI control group. These comparisons were made using unpaired t-tests.

RESULTS

Plasma omega-3 PUFA concentration and omega-3 to omega-6 PUFA ratio are negatively correlated with severity of chronic post-amputation pain in humans

To assess the relationship between omega-3 PUFA plasma levels and pain in the VIPER patients, we performed linear regressions of DHA, EPA, and their summed plasma levels versus S-LANSS severity score (Figure 1A,B). The analyses showed significant negative correlation between both individual or summed omega-3 levels and S-LANSS severity score. The same analysis was done using the omega-6 PUFAs arachidonic acid, linoleic acid, and n-6 docosapentaenoic acid (DPA n-6) (Figure 1C). No significant correlations were found between S-LANNS severity score and omega-6 PUFA levels. We were also interested in the relationship between the ratio of omega-3 to omega-6 PUFAs and pain in the VIPER patients experiencing pain (Figure 1D).

Linear regression revealed a significant negative correlation between omega-3 to omega-6 PUFA ratio and S-LANSS severity score.

Perioperative supplementation with DHA and EPA attenuates post-nerve injury mechanical allodynia in mice.

To test the effect of DHA and EPA supplementation with and without aspirin on mechanical allodynia in mice with surgically induced peripheral nerve injury, we created 4 groups of 10 mice each. One group underwent sham surgery and received daily control soybean oil. The remaining groups all underwent SNI surgery and received daily control soybean oil, DHA and EPA, or DHA, EPA, and aspirin.

We assessed the influence of omega-3 PUFA supplementation on post-operative allodynia by comparing the groups' mean paw withdrawal thresholds across all time points (Figure 2). As anticipated, the SNI control mice developed significant mechanical allodynia by POD3 ($p < 0.0001$) that peaked on PODs 9-12 and resolved by POD21. We found that treatment with DHA and EPA significantly attenuated the development of mechanical allodynia compared to SNI controls. The DHA/EPA group had improved allodynia compared to SNI on PODs 6, 9, and 15 ($>50\%$, $p < 0.05$), and trended towards improved outcome on POD12 (35.2%, $p = 0.055$). Similarly, the treatment group with aspirin had substantially improved allodynia compared to SNI on PODs 3 through 12 ($>50\%$, $p < 0.05$). Aspirin did not appreciably augment the therapeutic effect of omega-3 PUFA supplementation, as there were no significant differences between the two treatment groups. We also performed area under the curve and quadratic regression analyses of the paw withdrawal thresholds to confirm the effect of omega-3

supplementation on mechanical allodynia. These analyses corroborated the above results, indicating that omega-3 supplementation interfered with the development of robust allodynia seen in SNI controls. (See Supplementary Materials for details).

Perioperative supplementation with DHA and ASA may augment plasma protectin DX (PDX) and neuroprotectin D1 (NPD1) in mice. (Figure 3)

We next created groups of mice identical to those described above to test the effect of DHA, EPA, and aspirin supplementation on endogenous levels of pro-resolving oxylipins. Plasma samples collected from 5 mice per group on POD12 were analyzed using an LC-MS/MS assay.

There was a trend towards increased levels of the DHA-derived oxylipins protectin DX (PDX) and neuroprotectin D1 (NPD1) in the treatment group treated with aspirin compared to the SNI controls (116.4 ± 54.7 pg/mL and 90.3 ± 49.3 pg/mL; $p=0.07$ and $p=0.11$, respectively). There was no significant difference between the DHA/EPA treatment group and the SNI controls. Resolvin D1, resolvin D2, and maresin plasma levels were all below the assay's LLQ and could not be quantified.

DISCUSSION

Mechanistic understanding of the transition from acute to chronic neuropathic pain after nerve injury has advanced significantly over the past decade, but advances in preventive therapeutics continue to be slow. In response, there has been increasing focus since 2010 on public/private partnerships to advance preventive analgesic discovery by standardizing pre-clinical and clinical analgesic studies (19). Recent

evidence that DHA- and EPA-derived small lipid mediators such as neuroprotectin D1 can provide preventive analgesia in animal models of post-nerve injury pain has provided one of the most intriguing leads toward novel preventive therapeutics (6,20). The PRLMs themselves are short lived and difficult to produce in quantity, but supplementation with their omega-3 fatty acid precursors offers a possible way to increase the concentration of these small lipid mediators at the target injury site. In addition, aspirin acetylation of cyclooxygenase has been shown to increase the production of a group of aspirin derived PRLMs that may further increase the analgesic efficacy of DHA/EPA supplementation (5, 21).

In this study we found that the plasma concentrations of DHA and EPA in young, recent active duty military amputees negatively correlated with chronic post-amputation pain severity. It is unclear from this data whether reduced plasma DHA and EPA is a result of having already existing chronic pain or whether those patients with diets high in omega-3 fatty acids are less likely to develop severe chronic pain after surgery, but this intriguing correlation prompted us to study whether supplementation of DHA/EPA in mice undergoing peripheral nerve injury (including the nerve transection that occurs in amputation) would similarly reduce post nerve injury allodynia and whether this supplementation increased plasma PRLM levels. Therefore, we provided oral supplementation of DHA and EPA to mice before and after spared-tibial nerve injury and found that these injured mice had significantly reduced post-injury mechanical allodynia and a trend toward higher plasma neuroprotectin levels. This reduction in allodynia continued for 6 days after oral DHA/EPA supplementation was discontinued. By day 21 all four experimental groups returned to baseline. Though allodynia reduction

in the two DHA/EPA treatment groups was dramatic, it is difficult to conclude from this data whether supplementation acts as a therapeutic or as a preventive therapeutic due to the inherent improvement of mechanical allodynia over time in this particular peripheral nerve injury model. There are several models of peripheral nerve injury that produce more dramatic and long-lasting allodynia that could be used in future studies to verify that DHA/EPA supplementation is preventive and not just therapeutic (22).

To determine whether DHA/EPA supplementation led to measurable increases in plasma PRLMs, we collected blood plasma at post-operative day 12 from mice treated in an identical manner to the four groups tested for mechanical allodynia. After LC/MS separation and analysis, we found a trend towards higher plasma concentrations of two neuroprotectins, NPD1 and PDX. NPD1 has been well studied as an analgesic and neuroprotective agent and is one of the few compounds found to provide preventive analgesia in a mouse model of peripheral nerve injury (10,20,23). PDX has been less well studied in animal pain models, but has been shown to block neutrophil infiltration in a mouse model of peritonitis (24). Though plasma concentration changes of these two lipids did not reach significance (defined as p-value of 0.05), the trend toward neuroprotectin levels is intriguing. Future studies would include increased sample size and volume, along with analysis of injured peripheral tissues where these lipids are likely formed. Analysis of affected tissue with higher concentrations of these mediators may also allow evaluation of resolvin and maresin concentration changes with DHA/EPA supplementation, as the amounts of these PRLMs were below the lower limit of quantification in this study. Our study also included an aspirin treatment group since there are multiple aspirin-triggered PRLMs produced by the aspirin acetylated COX2

enzyme (5). However, aspirin treatment did not appear to enhance the reduction of mechanical allodynia in nerve-injured mice, and the effect of aspirin on the endogenous production of 18R-E series resolvins was not measurable since all resolvins in this study were present below the lower limit of quantification.

Though this pilot study did not definitively show that DHA/EPA supplementation increases plasma PRLMs, previous studies have demonstrated that supplementation with DHA and EPA translates to increased endogenous resolvin levels in healthy volunteers (21,25). Also, there is growing evidence that increasing the ratio of omega-3 to omega-6 fatty acids likely augments the beneficial effects of omega-3 PUFA supplementation on multiple disease states (26). Recent work by Ramsden et al supports the idea that dietary supplementation with omega-3 fatty acids increases the blood concentration of these small lipid mediators while reducing pain symptoms (27). This clinical trial concluded that migraine patients receiving a high omega-3 and low omega-6 fatty acid diet had significantly higher blood levels of the immediate precursors to resolvin and neuroprotectin biosynthesis and also increased resolvin D2 concentration. They also found that this dietary intervention reduced the incidence of migraine headache. Reducing omega-6 fatty acids in the mouse diet in addition to DHA/EPA supplementation may produce more dramatic improvements in mechanical allodynia and small lipid mediators. This will have to be evaluated in future preclinical studies.

Conclusions:

Our results suggest that perioperative DHA/EPA supplementation may provide a safe, inexpensive and effective way to increase pro-resolving lipid mediator levels and

prevent chronic pain in humans. Given the low-risk of side effects and current high prevalence of use, omega-3 PUFAs supplementation could easily be applied perioperatively. A larger preclinical trial with increased sample size and an added experimental group with low omega-6 fatty acid diet is needed to confirm these findings. A clinical trial in keeping with the FDA Critical Path initiative and ACTION committee goals would be an ideal next step after preclinical confirmation.

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Figure 1. Essential fatty acids and neuropathic pain in traumatic amputees

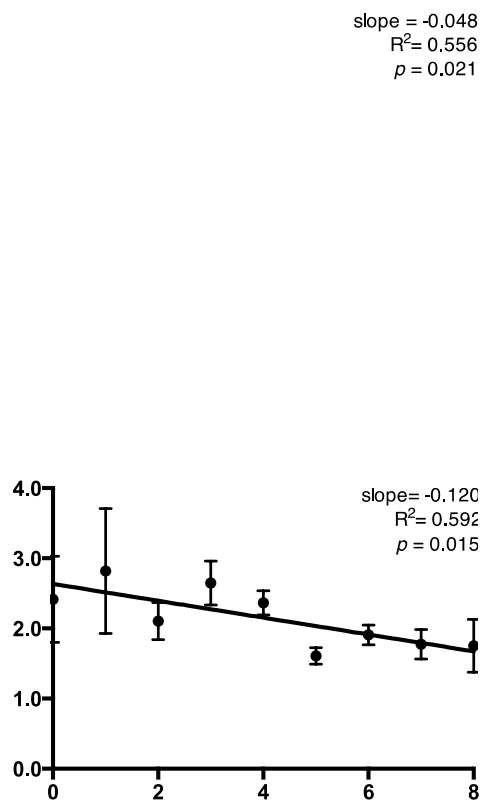


Figure 1: A-C: Plasma levels of the omega-3 fatty acids DHA and EPA are negatively correlated with neuropathic pain score in patients status-post traumatic amputation. Depicted are the summed or individual levels of the omega-3 polyunsaturated fatty acids (PUFAs) DHA and EPA in the plasma of 78 VIPER patients against the patients' S-LANSS severity score. Linear regression revealed significant negative correlation ($p < 0.05$) between plasma omega-3 PUFA levels and pain score. D: There was also a negative correlation between the ratio of omega-3 to omega-6 PUFAs and neuropathic pain score. The essential fatty acids levels are relative: they represent raw area counts from the LC/MS analyses that have been normalized such that the cohort has a median of 1.

Figure 2. Oral DHA/EPA supplementation reduces mechanical allodynia

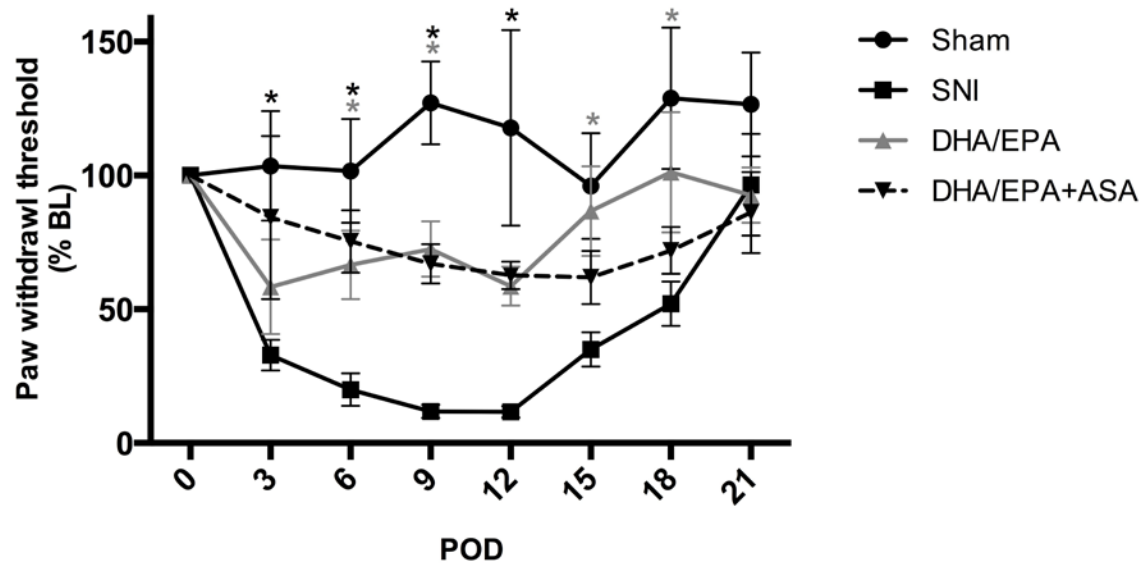


Figure 2: Perioperative supplementation with DHA and EPA attenuates mechanical allodynia following spared-nerve injury. Paw withdrawal thresholds are presented as mean percent of baseline \pm SEM, from baseline (POD0) through post-operative day (POD) 21. Lower thresholds represent mechanical allodynia. Mice treated with DHA and EPA (DHA/EPA, DHA/EPA + ASA) developed significantly less mechanical allodynia (50-60%) at the majority of time points compared to the spared-nerve injury control group (SNI). * represents significant difference of treatment group from SNI ($p < 0.05$).

Figure 3. Perioperative supplementation with DHA/EPA may increase plasma PLRMs

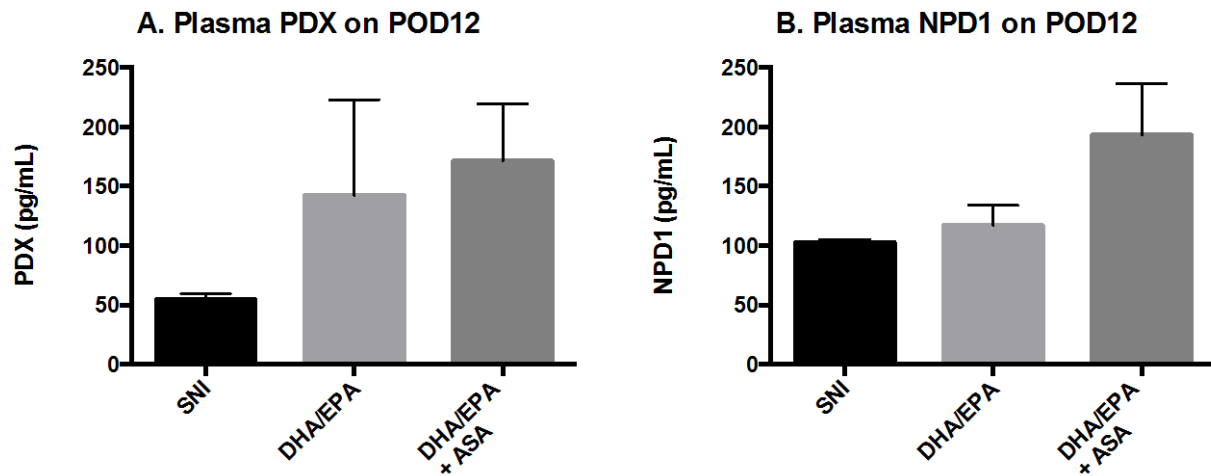


Figure 3: Perioperative supplementation with DHA, EPA, and aspirin may increase endogenous levels of neuroprotectins. A: Plasma levels of the neuroprotectin PDX trended towards increase in the DHA/EPA + ASA group compared to SNI controls on POD12 (+116.4, $p=0.07$). B: Plasma levels of neuroprotectin D1 (NPD1) trended towards an increase in the DHA/EPA + ASA group compared to SNI controls on POD12 (+90.3, $p=0.11$)

SUPPLEMENTARY MATERIAL

Statistical analysis

The areas under the “paw withdrawal threshold versus time” curve (AUC) were calculated using the trapezoid rule in Microsoft Excel and compared using a Kruskal-Wallis test with ad hoc multiplicity-corrected Dunn tests in Prism. Areas under curves have been used in clinical trials of pain as a summative measure of total pain relief and are customarily reported as percent difference from maximum AUC (28,29). For this experiment, AUC analysis is reported as mean percent of baseline paw withdrawal threshold AUC with SEM. It is thus used here as a summative measure of hindpaw sensitivity: lower AUCs correspond to increased mechanical allodynia, while those above 100% indicate lower sensitivity compared to baseline.

As another means of comparing the pain behavior of the different groups over time, centered quadratic polynomial regression analysis was performed on the behavioral data. Comparisons of best-fits were made using the Extra Sum of Squares F Test in a step-wise fashion: if comparison showed that the groups had significantly different fits, the most dissimilar group was removed from analysis until no significant difference in best fit was found between groups.

Results

Our AUC analysis showed that treatment with DHA and EPA reduced total mechanical allodynia over the course of this experiment (Figure 1B). Compared to the control SNI mice, the DHA/EPA and DHA/EPA and aspirin groups' percent baseline paw withdrawal thresholds were increased by 47.5% and 40.3%, respectively ($p < 0.05$). Again, there was no difference between the two treatment groups. This indicates that

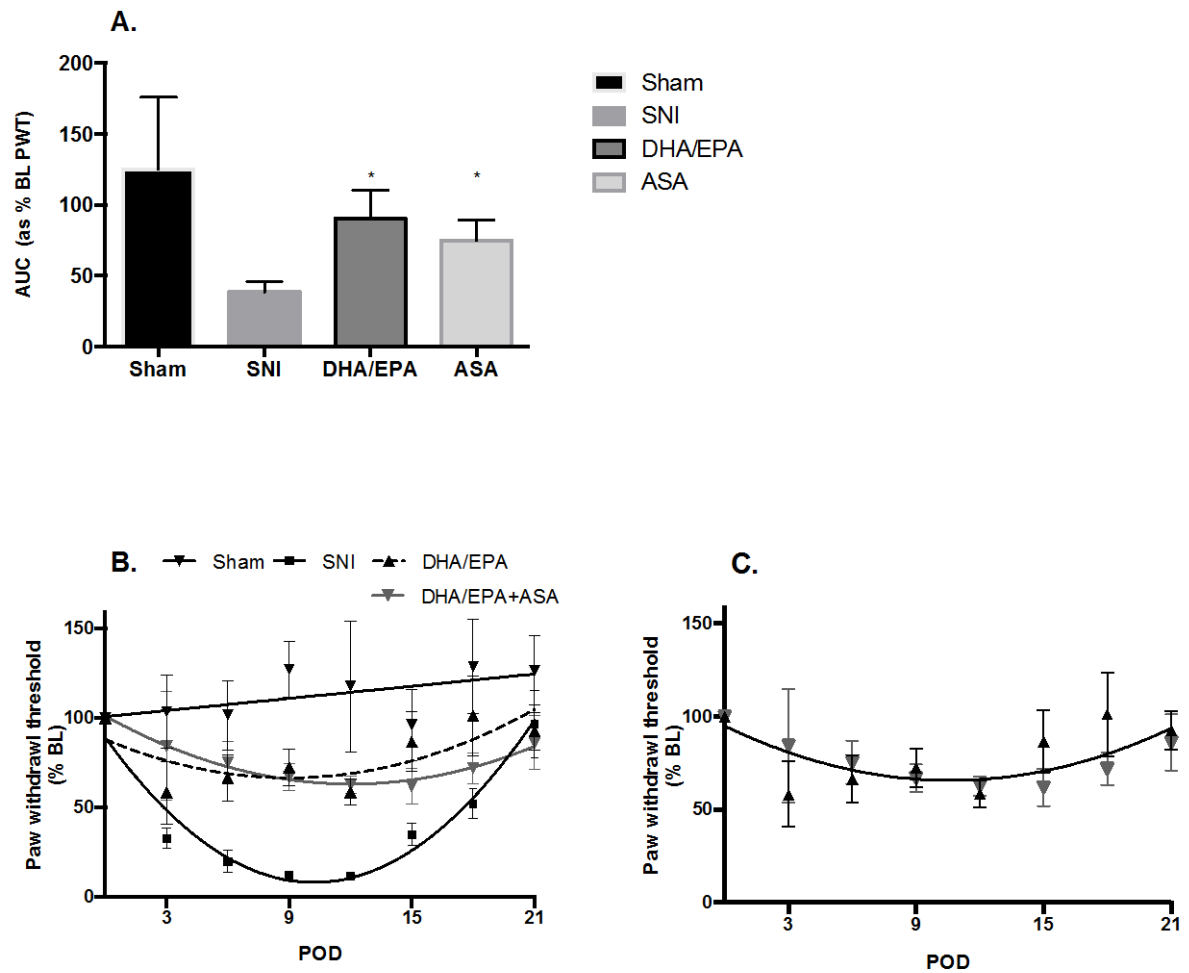
over the course of the experiment, those mice treated with DHA and EPA cumulatively experienced less mechanical allodynia after nerve injury compared to controls.

To better characterize and compare the pain behavior of the groups over time, we performed a centered quadratic polynomial regression analysis (Figures 1C-E). We found that a single model fit the data from the two treatment groups, and that this model differed significantly from the best-fit models for the sham and SNI control groups ($p < 0.0001$). The fits for the treatment and sham models were poor ($r^2 = 0.062$ and 0.014 , respectively), but the this regression analysis nevertheless illustrates that the mechanical allodynia that developed in the treatment groups was less robust and progressed differently following nerve injury than in controls.

Supplementary References

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Supplemental Figures



D.

DHA/EPA +/- ASA		Coefficient (SE)
$r^2 = 0.062$ MeanX= 10.27	B0	65.76 (5.30)
	B1	-0.14 (0.51)
	B2	0.26 (0.08)
SNI control		
$r^2 = 0.61$ MeanX=10.56	B0	8.25 (4.57)
	B1	0.55 (0.43)
	B2	0.78 (0.07)
Sham control		
$r^2 = 0.014$ MeanX=10.50	B0	112.7 (11.51)
	B1	1.135 (1.10)
	B2	0.0006 (0.08)

Supplemental figure captions:

A: AUC analysis: Area under the “paw withdrawal threshold versus time” curves (AUC) for the different groups are shown as median (+IQR) percent of baseline PWT; larger values represent higher PWTs and thus less mechanical allodynia over the course of the experiment. Compared to the control SNI mice, the DHA/EPA and DHA/EPA with aspirin groups’ percent baseline paw withdrawal thresholds were increased by 47.5% and 40.3%, respectively ($p < 0.05$).

B-D: Quadratic regression analysis: Comparisons of best-fits were made using the Extra Sum of Squares F Test in a step-wise fashion: if comparison showed that the groups had significantly different fits, the most dissimilar group was removed from analysis until no significant difference in best fit was found between groups. A single model fit the data from the two treatment groups, and this model differed significantly from the best-fit models for the sham and SNI control groups ($p < 0.0001$). Figure C shows the initial fits for all groups; figure D shows the final fit for the two treatment groups. Figure E provides the model parameters.

ACUTE & PERIOPERATIVE PAIN SECTION

Original Research Article

Pain Phenotypes and Associated Clinical Risk Factors Following Traumatic Amputation: Results from Veterans Integrated Pain Evaluation Research (VIPER)

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or techniques named in the report. The manuscript was prepared in its entirety by the authors noted above.

Authors Drs Buchheit and Van de Ven contributed equally to this manuscript.

Abstract

Objective. To define clinical phenotypes of postamputation pain and identify markers of risk for the development of chronic pain.

Design. Cross-sectional study of military service members enrolled 3-18 months after traumatic amputation injury.

Setting. Military Medical Center

Subjects. 124 recent active duty military service members

Methods. Study subjects completed multiple pain and psychometric questionnaires to assess the qualities of phantom and residual limb pain. Medical records were reviewed to determine the presence/absence of a regional catheter near the time of injury. Subtypes of residual limb pain (somatic, neuroma, and complex regional pain syndrome) were additionally analyzed and associated with clinical risk factors.

Results. A majority of enrolled patients (64.5%) reported clinically significant pain (pain score ≥ 3 averaged over previous week). 61% experienced residual limb pain and 58% experienced phantom pain. When analysis of pain subtypes was performed in those with residual limb pain, we found evidence of a sensitized neuroma in 48.7%, somatic pain in 40.8%, and complex regional pain syndrome in 19.7% of individuals. The presence of clinically significant neuropathic residual limb pain was

associated with symptoms of PTSD and depression. Neuropathic pain of any severity was associated with symptoms of all four assessed clinical risk factors: depression, PTSD, catastrophizing, and the absence of regional analgesia catheter.

Conclusions. Most military service members in this cohort suffered both phantom and residual limb pain following amputation. Neuroma was a common cause of neuropathic pain in this group. Associated risk factors for significant neuropathic pain included PTSD and depression. PTSD, depression, catastrophizing, and the absence of a regional analgesia catheter were associated with neuropathic pain of any severity.

Key Words. Amputation; Nerve Injury; Residual Limb Pain; Phantom Pain; Neuroma; Regional Catheter; Depression; Catastrophizing; Post-traumatic Stress Disorder

Introduction

Chronic pain after trauma and surgery has a direct and lasting impact on the quality of life of thousands of injured military service members, including the over 1,573 individuals suffering battlefield amputation injury since 2001 [1]. In addition, more than 100,000 patients undergo amputation each year in the United States due to trauma or medical conditions including diabetes and peripheral vascular disease [2,3]. A significant percentage of these patients will suffer long-term morbidity from chronic pain with an incidence ranging from 50 to 80% [4,5].

The treatment of persistent, residual limb or phantom limb pain with existing analgesics has proven difficult [6] as is true for most types of persistent neuropathic pain. This failure has produced growing interest in identifying strategies to prevent chronic postsurgical pain (CPSP) of all types [7]. Unfortunately, most prospective, blinded and randomized trials using regional analgesia or perioperative pharmacologic analgesics have failed to show significant efficacy [8,9]. Promisingly, however, two recent Cochrane systematic reviews concluded that, when these trials are combined in a meta-analysis, some types of perioperative regional analgesia and pharmacologic analgesic therapy may indeed reduce the risk of chronic pain after surgery. Nonetheless, the overall efficacy was described as “modest” [10,11]. The reasons behind the “modest” and equivocal ability of these preventive strategies to reduce the incidence of CPSP are unknown. Several contributing factors have been considered including inadequate classification of chronic pain syndromes and the inability to identify patients most at risk of CPSP [12–14].

The importance of systematic evaluation of symptoms and signs in pain medicine has been highlighted in

recent years [15]. Diagnostic enhancements have led to improvements in the treatment of medical illness such as leukemia and lymphoma [16], and granular analysis of disease subtypes is increasingly believed to be pivotal if we are to develop personalized pain therapies [17,18]. Though methods for clinical diagnosis in neuropathic pain have improved [19,20], significant limitations continue to exist, especially in the diagnostic classification of postamputation pain subtypes. Although neuroma is frequently observed and complex regional pain syndrome has been described after amputation, these separate entities are often lumped together as one diagnostic entity [21,22]. These separate subtypes likely result from distinct pathophysiologic mechanisms, may be associated with unique biomarkers and likely require unique approaches to treatment and prevention. For instance, a patient with a painful residual limb secondary to infection, heterotopic ossification or a poorly fitting prosthesis will require a significantly different therapy than a patient with a sensitized neuroma.

Identifying effective preventive therapies for amputation and surgical nerve injury requires an understanding of predisposing clinical factors [2,23,24]. Studies attempting to identify psychosocial variables as risk factors or correlates of pain have become more numerous in the past decade, but results have been inconsistent [25]. Nonetheless, there have been studies associating psychological conditions such as post-traumatic stress disorder (PTSD), depression, and pain catastrophizing with chronic pain [26–28]. The identification of clinical factors that predispose patients to CPSP is critical for adequate patient risk stratification and will increase the power of future trials to identify preventive therapies.

We report here results from VIPER (Veterans Integrated Pain Evaluation Research), a study of 124 recent active military traumatic amputees designed to discriminate pain phenotypes, evaluate clinical risk factors, discover biomarkers of chronic pain, and identify novel pain pathways. Subjects were enrolled three to 18 months after amputation, and extensive questionnaires were completed, assessing demographic data, surgical procedure, and screening for post-traumatic stress disorder (PTSD), depression, and pain catastrophizing. In order to more precisely categorize the distinct pain conditions occurring after amputation we used a diagnostic algorithm, the Duke postamputation pain algorithm (DUKE-PAPA), modified from our original algorithm [29], to differentiate the subtypes of postamputation pain. Using this phenotypic data and algorithm-based grouping of patients into diagnostic subgroups, we attempted to determine whether PTSD, depression, and pain catastrophizing were associated with chronic pain or the predetermined pain subtypes following amputation.

Given the equivocal literature on pain prevention with peripheral nerve catheters [30,31], we additionally evaluated the incidence of chronic pain after amputation in

patients with and without peripheral nerve catheters placed soon after the time of injury.

Methods

Study Design

The Defense and Veterans Center for Integrative Pain Management (DVCIPM – DVCIPM.org) obtained Institutional Review Board approval at Walter Reed National Military Medical Center (WRNMMC) to enroll 124 recent active duty military post-traumatic amputees receiving care 3–18 months after amputation. We implemented a 3-month minimum given that chronic pain is usually defined as lasting 3 months or greater in most study populations. An 18-month cutoff was employed as the time-course of recovery/rehabilitation at WRNMMC is usually complete by this point in time, and few military service members would be present and eligible for enrollment after this period. Multiple pain and psychometric questionnaires were administered to each individual and completed with minimal guidance by a DVCIPM healthcare provider.

Subjects were included if they were a military health care system beneficiary aged 18 years or older and undergoing treatment at WRNMMC with a diagnosis of postinjury amputation of all or part of one limb. Amputation injury must also have occurred between 3 and 18 months prior to enrollment.

Patients were excluded if they were afflicted with severe traumatic brain injury, significant cognitive deficits, substantial hearing loss, spinal cord injury with permanent or persistent deficits, ongoing tissue damage that might cause pain, infection, heterotrophic ossification, poorly fitting prosthesis, or hip disarticulation.

Data Collection

From November 2011 to July 2013, demographic data were collected and multiple previously validated questionnaires were administered to quantify and qualitatively stratify each subject's pain. We utilized the Brief Pain Inventory (BPI) Short Form for assessment of pain and function [32], the Defense Veterans Pain Rating Scale (DVPRS) for additional symptomatic evaluation given its validity in this patient population [33], the Self-Report Leeds Assessment of Neuropathic Symptoms and Signs (S-LANSS) to discriminate between nociceptive and neuropathic pain [34], Post Traumatic Stress Disorder Checklist-Military (PCL-M) normalized for a military population to screen for PTSD [35], Pain Catastrophizing Scale (PCS) to assess catastrophizing and its subscales of rumination, magnification and helplessness [36], Pain Health Questionnaire-9 (PHQ-9) to detect symptoms of depression [37], and Complex Regional Pain Syndrome (CRPS) Questionnaire using the Budapest Clinical Criteria. In the absence of a validated assessment tool for the detection of CRPS in an amputation population, we chose to apply the

“Budapest Criteria” that is commonly used in existing-limb populations. Given that physical exam findings and therefore sensitivity of detection of CRPS in a missing-limb population might be diminished, we utilized the more sensitive “clinical” criteria as our detection tool [38]. Phantom sensation, phantom pain, residual limb pain and the presence of a sensitized neuroma were assessed through questions from the Groningen Questionnaire Problems After Arm/Leg Amputation [4] (Table 1).

A physical exam was also performed at the time of assessment, documenting allodynia/hyperalgesia, presence/absence of a sensitized neuroma (Tinel's sign), temperature/color asymmetry, wound status, edema and skin/hair changes outside of the injury. The use of regional anesthesia catheter infusion was determined through a comprehensive review of each patient's chart from the time of traumatic injury and through the period of subsequent care. Although we were able to confirm catheter placement through review of records, many of these catheters were placed under battlefield conditions, and we were not able to always confirm the exact location of catheter placement. We therefore do not report on this finding.

Pain and Assessment Tool Interpretation

We defined clinically significant pain (cases) as an average pain score over the past week of greater than or equal to 3/10 on a numeric rating scale (NRS). This cut-off value has been used in previous pain literature to distinguish “mild” pain from “moderate/severe” pain, and has been used in prior studies of postamputation pain [8,38,39]. Those patients with clinically significant pain were further adjudicated into pain subtypes. Those subjects with pain less than 3/10 but greater than 0/10 completed all the data collection questionnaires, but subtypes were not analyzed. This case/control methodology was chosen to facilitate our parallel analyses where we are using case status to correlate with genetic polymorphisms and biomarkers of risk in susceptible individuals.

To examine potential, associated psychological comorbidities we defined diagnostic cutoff values for the assessment instruments that we utilized. Pain catastrophizing was defined as a PCS score of 15 or greater based on the work of Sullivan et al. [3], and consistent with other studies of risk factors for chronic postsurgical pain [40]. We defined possible depression as a PHQ-9 score of 10 or greater based on a meta-analysis by Manea et al. who found PHQ-9 scores between 8 and 11 were able to detect depression with reasonable sensitivity and specificity [41]. The presence of possible PTSD was defined by a PCL-M score of 50 or greater, normalized for a military population [42].

Pain Subtype Adjudication

Between May 15 2012 and June 16 2014, three or more members of the VIPER Adjudication Committee

Table 1 Assessment tools

Symptom	Assessment Tool
Pain and function	Brief Pain Inventory (BPI) Short Form
Pain and function	Defense Veterans Pain Rating Scale (DVPRS),
Neuropathic symptoms	Self-Report Leeds Assessment of Neuropathic Symptoms and Signs (S-LANSS),
Post-traumatic stress disorder	Post-traumatic Stress Disorder Military Checklist (PCL-M)
Pain Catastrophizing	Pain Catastrophizing Scale (PCS),
Depression	Pain Health Questionnaire-9 (PHQ-9)
Complex Regional Pain Syndrome	Budapest Complex Regional Pain Syndrome (CRPS) Questionnaire
Phantom sensation, phantom pain, residual limb pain, neuroma	Questions from Groningen Questionnaire Problems After Leg Arm Amputation

(composed of Drs. Buchheit, Vandeven, Buckenmaier, Hsia, Macleod, and Shaw) met on six occasions to review data and adjudicate the study subjects into pain subtype using the previously described Duke-PAPA algorithm (Figure 1). This algorithm systematically characterizes postamputation pain into multiple discrete phenotypes that include phantom pain and subtypes of residual limb pain such as somatic, neuroma, complex regional pain syndrome, and mosaic (not otherwise specified) [29].

The adjudication process started with classifying study subjects into cases and controls. At the time of assessment, if the patient experienced an average pain score over the past week of greater than or equal to 3/10 on a numeric rating scale, they were considered a “case” and were therefore adjudicated into pain subtypes. The presence/absence of phantom and residual limb pain were then assessed using a subset of questions from the Groningen assessment [4]. Phantom pain was distinguished from nonpainful phantom sensation, and defined by a positive response to the following question: “do you experience pain in any part of the amputated arm and/or leg?”. Residual limb pain was defined by a positive response to the question: “do you have any pain in the stump?”. If residual limb pain was present, it was then classified as “somatic” or “neuropathic” using the S-LANSS questionnaire with a cutoff value of 12 or greater defining pain as neuropathic. Those with neuropathic pain were then further characterized as having either neuroma or complex regional pain syndrome (CRPS) using both questionnaire and physical exam data. Pain elicited by tapping pressure applied at a specific point (Tinel’s sign) or patient reported pain with focal point pressure was defined as neuroma. Budapest Clinical Criteria were used to define CRPS, requiring three out of four clinical symptoms and two out of four physical exam findings. If patients had an SLANSS score of 12 or greater but did not meet diagnostic criteria for CRPS or neuroma, the patient was classified as experiencing a not otherwise specified “mosaic neuralgia.”

All patients with pain greater than 0/10 were evaluated with the assessment tools noted above. Those with pain less than 3/10 were considered part of the

“control” cohort for future biomarker analysis. These subjects were not adjudicated into pain subtypes but were included along with the rest of the study sample in an exploratory analysis examining risk factors for post-amputation pain. Pain was considered “severe” if NRS pain score > 5.

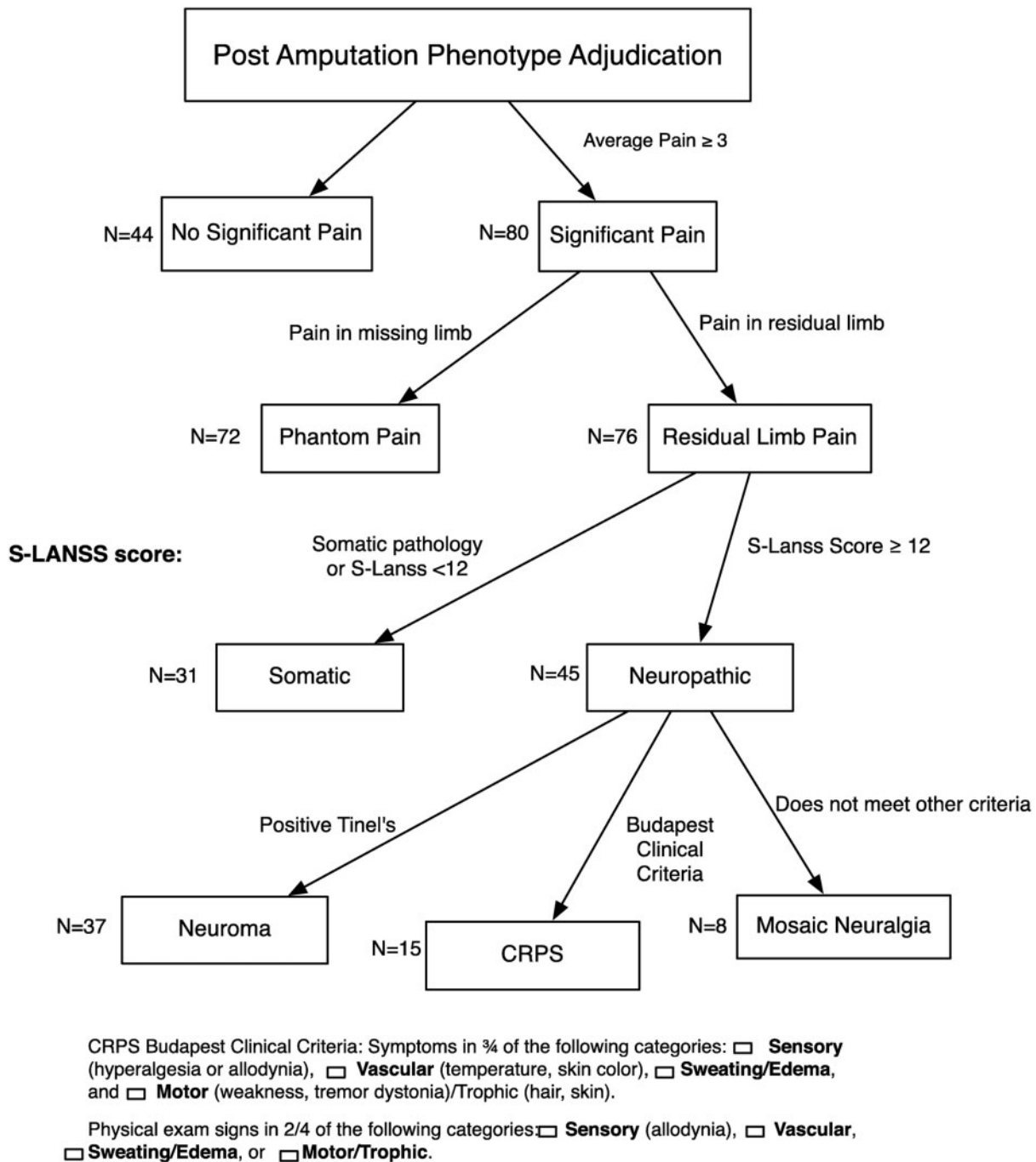
Statistical Analysis

All questionnaire data were entered into the Research Electronic Data Capture (REDCap)TM database and adjudication data were entered into a spreadsheet that was stored on the Duke secure servers only accessible by research staff. Data analysis was performed using SAS version 9.3 (SAS Institute Inc., Cary, NC) and R (version 3.1.2) statistical software programs. For the patient demographic data, the Wilcoxon rank sum test was used for the analysis of continuous variables and the Pearson’s chi-square test was used to analyze categorical variables. For the clinical risk factors and chronic pain subtypes, odds ratios with 95% confidence intervals were calculated for each categorical variable and statistical significance was calculated with Fisher’s exact test or the likelihood ratio chi-squared test. *P* values from Fisher’s exact test are presented where appropriate (any expected cell counts < 5 in contingency tables). A *P* value < 0.05 was considered significant for all analyses. No adjustments were made for multiple testing in this exploratory investigation.

Results

Patient Demographics and Pain Incidence

The demographic characteristics of patients defined as cases versus those defined as controls are reported in Table 2. There were no significant differences in age, body mass index, time as amputation, ethnicity, amputation site or presence of regional analgesia catheter around the time of amputation between patients who had clinically significant pain at the time of enrollment compared to those who did not. When we analyzed the demographic characteristics of those who received or did not receive a regional analgesia catheter, we observed no difference.



No Pain Phantom RLP Somatic RLP Neuroma RLP CRPS RLP Mosaic

Figure 1 Phenotype adjudication algorithm.

A majority of enrolled patients (80/124 or 64.5%) reported clinically significant pain scores (pain score ≥ 3 averaged over the previous week, Table 3) while 17% of patients reported experiencing severe pain (pain

score > 5). The distribution of patients noting clinically significant pain and severe pain in this cohort is consistent with previously published studies of chronic pain after amputation [4,5].

Table 2 Patient demographic data

Demographic	Control (N = 44) Mean (SD)	Case (N = 80) Mean (SD)	P value
Age	25.4 (5)	26.9 (6.8)	0.1562*
Body Mass Index	26.1 (3.6)	26.6 (3)	0.2345*
Time since amputation (months)	8.4 (3.9)	8.9 (5.2)	0.9457*
Male	N (%) 44 (100)	N (%) 78(97.5)	0.9577^
Regional catheter	22 (50)	31 (38.8)	0.2260^
Ethnicity	N (%)	N (%)	0.5700^
American Indian/Alaska Native	0 (0)	2 (2.5)	
Asian	2 (4.5)	1 (1.3)	
Native Hawaiian or Other Pacific Islander	0 (0)	1 (1.3)	
Black or African American	3 (6.8)	5 (6.3)	
White	39 (88.6)	70 (88.6)	
Amputation Site	N (%)	N (%)	0.8478^
Left upper extremity	2 (4.5)	5 (6.3)	
Right upper extremity	1 (2.3)	3 (3.8)	
Left lower extremity	22 (50)	34 (42.5)	
Right lower extremity	19 (43.2)	38 (47.5)	

*P value generated from Wilcoxon Rank-Sum test. ^P value generated from chi-squared test.

Table 3 Distribution of pain subtypes

Pain Phenotypes	
Total Patients Enrolled	All Patients n=124
Any Patient with Pain (NRS>0)	Any Pain n=115 (92.7%)
Patients with Significant Pain (NRS≥3)	All Significant Pain n=80 (64.5%)
Residual Limb Pain (NRS≥3)	Significant Residual Limb Pain n=76 (61%)
Phantom Limb Pain (NRS≥3)	Significant Phantom Limb Pain n=72 (58%)
Phenotypic Subtypes of Significant Residual Limb Pain	
Neuroma (n=37, 48.7% of RLP)	2 +35 Neuroma
Somatic (n=31, 40.8% of RLP)	4 +27 Somatic
CRPS (n=15, 19.7% of RLP)	CRPS n=15
Mosaic (n=8, 10.5% of RLP)	2 +6 Mos

Subtypes of Chronic Postamputation Pain

Of the 80 subjects who described significant postamputation pain (NRS $\geq 3/10$), the majority experienced both residual limb pain (76/80) and phantom limb pain (72/80) (Table 3). Analysis of significant residual limb pain subtypes revealed that 37/76 (48.7%) demonstrated evidence of a sensitized neuroma. Symptoms and exam

evidence of complex regional pain syndrome was observed in 15/76 (19.7%). It was notable that all subjects with CRPS also had evidence of a symptomatic neuroma. Somatic pain was identified in 31/76 patients (40.8%) with residual limb pain. Only eight patients (10.5%) with neuropathic residual limb pain could not be classified as having either neuroma or CRPS and received the mosaic neuralgia classification.

Association Between Clinical Risk Factors and Subtypes of Chronic Postamputation Pain

Each enrolled patient completed PHQ-9, PCL-M, and PCS questionnaires assessing the presence of depression, post-traumatic stress disorder (PTSD) and pain catastrophizing, respectively. Odds ratios were calculated for these clinical risk factors in association with chronic postamputation pain (see Table 4).

When we analyzed subjects with clinically significant residual limb pain (RLP) (NRS $\geq 3/10$, N=76), without regard to subtype, no associations with clinical risk factors were observed. However, when neuropathic RLP was analyzed (N=45), significant associations were noted with PTSD and depression. When we further discriminated subtypes of neuropathic RLP, we found that the RLP subtypes of neuroma (N=37) and CRPS (N=15) were additionally associated with PTSD and depression. Non-neuropathic residual limb pain (somatic RLP) was negatively associated with the presence of PTSD only.

Exploratory Analysis of the Association Between Clinical Risk Factors and Postamputation Pain

Because there were multiple patients with some degree of neuropathic pain in the control group that we did not want to completely exclude, we conducted an exploratory analysis examining risk factors for postamputation pain in all subjects. When all patients (NRS $> 0/10$) with neuropathic symptoms (SLANSS ≥ 12) were included,

we found neuropathic pain to be significantly associated with all four clinical risk factors (catastrophizing, PTSD, depression, and regional analgesia catheter) (see right hand column of Table 4). Thus, for the study sample as a whole, patients who met criteria for PTSD, who scored higher on depression or pain catastrophizing, or had the absence of a regional analgesia catheter were much more likely to report higher levels of neuropathic pain.

Additional analysis of opioid pain medication (oxycodone, morphine, hydrocodone, methadone, and fentanyl) use was performed. There were 30 study subjects that were taking an opioid at the time of assessment. As one might anticipate, patients who were cases (average pain ≥ 3) were more likely to use opioids (27/30, 90%) than those who were controls (3/30, 10%).

Discussion

The Veterans Integrated Pain Evaluation Research (VIPER) study included young, active duty soldiers who suffered traumatic battlefield amputation within 3–18 months of enrollment. The VIPER study was designed to distinguish pain subtypes, analyze associations between pain and a number of clinical risk factors, and discover novel biomarkers unique to each pain subtype. Relative youth and lack of medical comorbidity makes this patient population unique. This relatively homogeneous patient population may also provide a less noisy background when comparing genetic, epigenetic, and proteomic signatures between patients with and without

Table 4 Clinical risk factors for development of postamputation chronic pain subtypes

	Chronic Pain (cases) N = 80	Phantom Pain (cases) N = 72	RLP (cases) N = 76	RLP-Somatic (cases) N = 31	RLP-Neuropathic (cases) N = 45	RLP-Neuroma (cases) N = 37	RLP-CRPS (cases) N = 15	RLP-Mosaic (cases) N = 8	Neuropathic Pain (All) N = 61
	OR (CI) [P]	OR (CI) [P]	OR (CI) [P]	OR (CI) [P]	OR (CI) [P]	OR (CI) [P]	OR (CI) [P]	OR (CI) [P]	OR (CI) [P]
PCS	13.39 (1.73–103.87)	6.20 (0.34–112.49)	3.05 (0.16–59.29)	0.48 (0.15–1.50)	2.71 (0.87–8.45)	2.47 (0.85–7.14)	1.82 (0.53–6.20)	1.08 (0.20–5.85)	3.78 (1.28–11.18)
[P value]	[0.0016]*	[0.1876]	[0.5676]	[0.194]	[0.0735]	[0.0899]	[0.3474]	[1.0000]	[0.0103]*
PCL-M	10.08 (1.28–79.14)	1.72 (0.20–15.14)	2.31 (0.12–45.15)	0.19 (0.04–0.89)	6.92 (1.44–33.17)	6.67 (1.71–26.04)	7.13 (1.98–25.70)	0.58 (0.07–5.12)	9.28 (2.01–42.88)
[P value]	[0.0099]*	[1.0000]	[1.0000]	[0.0373]*	[0.0088]*	[0.0038]*	[0.0029]*	[1.0000]	[0.0010]*
PHQ-9	2.40 (0.94–6.13)	9.13 (0.51–164.62)	4.46 (0.23–86.00)	0.38 (0.13–1.09)	3.53 (1.22–10.19)	3.72 (1.36–10.15)	4.59 (1.42–14.91)	0.71 (0.13–3.79)	4.46 (1.81–10.99)
[P value]	[0.055]	[0.0520]	[0.3036]	[0.0624]	[0.0142]*	[0.0081]*	[0.010]*	[1.0000]	[0.0006]*
Regional Catheter	0.63 (0.30–1.33)	5.00 (0.58–42.80)	1.96 (0.19–19.70)	1.24 (0.50–3.12)	0.91 (0.37–2.25)	1.42 (0.58–3.51)	2.09 (0.67–6.49)	0.20 (0.02–1.71)	0.44 (0.21–0.92)
[P value]	[0.227]	[0.142]	[1.0000]	[0.6423]	[0.8397]	[0.4443]	[0.2034]	[0.142]	[0.0269]*

The odds-ratios for development of chronic pain (all types), neuropathic pain, phantom limb pain, all types of residual limb pain, somatic residual limb pain, residual limb pain from presence of neuroma, complex regional pain syndrome, mosaic neuropathic residual limb pain and all neuropathic residual limb pain are reported above with P values in brackets. Factors associated with significant risk of a specific pain subtype (P value < 0.05) are marked with an asterisk and italicized in bold. The data presented in the final column include total patients with neuropathic pain regardless of case or control status.

chronic residual limb pain as we attempt to identify biomarkers of pain susceptibility in the future.

Data on pain characteristics were collected using four distinct pain evaluation scales for each enrolled patient including a visual analogue scale (VAS) score, S-LANSS score for evaluation of neuropathic pain, BPI, and DVPRS. DVPRS and VAS are well-validated pain measurement tools which record pain.

Using the average pain score over the past week as our method to categorize patients as either control (<3) or case (≥ 3), we found that the overall incidence of significant chronic pain after amputation to be 65%, agreeing well with previously published data [2,43–45]. We additionally observed that 90% of patients receiving opioid analgesics at the time of study enrollment described significant pain (≥ 3), and therefore, were analyzed as cases. Given that only 3/10 (10%) of those receiving opioid medications were part of the control group (average pain <3), these medications appear to have minimally affected the case/control status of study subjects.

Pain phenotype adjudication using the Duke-PAPA algorithm [29] revealed that the majority of amputees with significant chronic pain were experiencing both phantom limb pain sensations and residual limb pain (68 out of 80). Almost 60% of patients with residual limb pain were determined to have chronic neuropathic pain (45 out of 76) as defined by the S-LANSS scoring system while the remaining 40% were found to have somatic pain in the residual limb.

Although the coexistence of phantom and RLP has been noted previously, it has not been reported to the degree observed in this cohort [5,43]. Literature in recent decades has increasingly supported central causes of phantom pain as a dominant paradigm, with decreasing emphasis on the role of peripheral neurologic input in maintaining phantom limb pain [46,47]. However, the peripheral contributions to phantom limb pain have again been questioned with the publication of successful treatment of phantom pain with intraforaminal blockade of the dorsal root ganglion [48]. The strong diagnostic coexistence of sensitized neuromas, residual limb pain, and phantom pain in this study further supports a likely important interplay between these pathologic processes. However, we must also exercise caution, as causation cannot be implied from this observational study.

It is also notable that there is a high prevalence of neuromas in this postamputation population (48.7% of those with residual limb pain had evidence of a neuroma). This prevalence is higher than previously reported incidences [5,49] and further strengthens the importance of neuroma in the comprehensive treatment of the patient with postamputation pain.

Though previous epidemiological analysis of CRPS patients showed that 24% of CRPS patients had surgery as the inciting injury [50], and CRPS has been

observed after amputation [6,51] it is unclear how many postsurgical patients develop CRPS after amputation or other surgical procedures involving injury to a major peripheral nerve. Using the Budapest Criteria, we found 15 out of 76 (19.7%) patients with chronic residual limb pain were defined as having CRPS in this cohort. Although significantly less common than neuroma in our study, we believe it is important to distinguish this diagnostic group given the therapeutic treatment implications.

In the past 10 years, multiple chronic postsurgical pain studies have also collected data on patient psychosocial variables as potential risk factors for development of pain [25,52]. Many of these have shown a positive correlation between the presence of CPSP and depression, anxiety, PTSD, and pain catastrophizing [53,54]. In patients experiencing chronic postmastectomy pain, for example, anxiety and pain catastrophizing were significantly associated with the presence of pain [55]. There are very few studies, however, specifically looking at an association between the presence of pain and PTSD, depression or pain catastrophizing in amputees [44,56]. Additionally, it is unknown if there are stronger associations with any of the subtypes of postamputation pain.

In our study, we found a significant association between symptoms of catastrophizing and PTSD and clinically significant ($\text{NRS} \geq 3/10$) chronic postamputation pain. It is notable that when we analyzed risk factors for cases of residual limb pain without regard to subtype discrimination, we found no significant associations. However, when we examined the risk factor associations for the defined subtypes of residual limb pain, we found significant associations between PTSD and depression and all three subtypes of neuropathic RLP (RLP neuropathic, RLP neuroma, RLP CRPS). These findings imply that risk factors (and therefore potentially treatments) do not affect all pain conditions equally. Interestingly, in an exploratory analysis that included all study patients (including those with $\text{NRS} < 3$) we found that all four risk factors were related to neuropathic pain. Taken together, these findings suggest that risk factors such as psychosocial variables and regional analgesia catheter placement may play a role in postamputation pain.

As all data were collected at one time point months after amputation, it is not possible to determine whether these psychosocial factors were present before surgery (acting as risk factors for development of pain) or if they developed subsequent to injury as a result of the traumatic experience. There is some evidence that pre-operative depression and anxiety are associated with a higher incidence of pain after surgery. Brander et al. assessed pre-operative depression and anxiety in patients about to undergo total knee replacement and found a significant increase in chronic pain incidence at one year in patients with high Beck-Depression Inventory and State-Trait Anxiety Index scores [57]. A number of other studies have assessed psychosocial variables as predictive risk factors but most have been

in patients undergoing lumbar spine surgery [58,59]. Both total knee arthroplasty patients and lumbar spine surgery patients often have a long history of chronic pain before surgery so it is unclear whether these findings can be applied to CPSP that occurs in patients without pre-existing pain conditions.

There have been multiple previous attempts to use peri-operative regional and neuraxial analgesia to prevent chronic postamputation pain. After some initial encouraging results [60–62] a number of small randomized trials of short term regional anesthesia did not show any effect on chronic phantom or residual pain incidence after amputation [8,31,63]. It is unclear if this lack of effect was due to underpowered studies, incomplete clinical pain syndrome classification, or the technique and duration of therapy. In our unique patient cohort, consisting of young men and women experiencing limb trauma and amputation, we found that patients who had a documented placement of peripheral nerve catheters near the time of traumatic injury were less likely to have neuropathic pain symptoms in the affected limb or stump. The presence of a peripheral nerve catheter at the time of injury or amputation was determined through an extensive review of each patient's military medical record. It is also interesting to note that in this cohort, the median duration of catheter treatment was 10 days with a maximum catheter duration of 29 days, considerably longer than many other studies of regional analgesia catheters [64]. This extended duration of catheter infusion may have a beneficial effect as has been previously demonstrated by Borghi et al. [65]. As understanding of the extended inflammatory response leading to nociceptor sensitization advances [66–71], and the importance of preventing abnormal nociceptor signaling throughout the course of this inflammatory response becomes more clear [72–76], it is intriguing that patients with a prolonged exposure to local anesthetics around a peripheral nerve may have a decreased incidence of neuropathic pain after amputation.

There are multiple limitations to this research. First of all, our small sample size (124 subjects) limits the analysis and conclusions, particularly in regards to the correlations between neuropathic pain subtypes, clinical risk factors, and the use of a regional analgesia catheter. Answering these questions would require either a larger study or a more focused clinical question. We also have limitations with our regional analgesia catheter use given the lack of more granular data on catheter location and drug doses, although it is reasonable to assume that the majority of these catheters were placed in proximity to the target nerves under ultrasound guidance. These are inherent challenges in the collection of information in the setting of active military conflict. The undefined role of traumatic brain injury (TBI) in this research cohort might also be considered a limitation of this study. Although severe TBI was an exclusion criterion, many of the service members most likely experienced mild to moderate TBI, affecting the experience of pain. A granular description of all comorbidities would have been

ideal to capture in this population. We were acutely aware throughout the course of the study to avoid additional disruption in the lives of the service members and the negative effects of questionnaire burden. We therefore gathered only the most critical data necessary to accomplish the aims of this research. It is reasonable to assume that the majority of those injured experienced a mild to moderate TBI, maintaining a relatively homogeneous sample.

Additional limitations would include the lack of pre-injury pain assessment and longitudinal follow-up of study subjects. We do not know if any of the study subjects had pre-existing chronic pain conditions that might affect outcomes following traumatic injury. It is also unclear if pain severity, quality, and phenotype might evolve over the subsequent months in these military service members, especially in those enrolled earlier in their recovery (3–6 months).

Conclusions

In conclusion, the discrimination of pain subtypes in this observational study of young, healthy traumatic amputees reveals a similar prevalence of postamputation pain with current literature [21], but a significant phenotypic overlap of both phantom and residual limb pain. There is additionally a prominent representation of sensitized neuromas as a cause of chronic residual limb pain, identifying this condition as a potential target for future therapies. Utilizing the Duke-PAPA diagnostic algorithm, we were able to classify patients into known categories of pain syndromes including phantom and residual limb pain, and further into RLP subtypes such as somatic, neuroma, complex regional pain syndrome, and mosaic neuralgia. Furthermore, we were able to demonstrate a significant association between symptoms of PTSD and depression and all subtypes of neuropathic RLP. Though presence of a peripheral nerve catheter did not reduce the number of patients with significant neuropathic RLP, it did reduce the incidence of neuropathic pain of all severities. These risk factors did not associate as strongly with somatic pain syndromes, consistent with a distinct pathophysiology. From this work it is unclear whether these clinical factors are predictive of future chronic pain development. Future longitudinal studies are needed to examine this question, and an ideal intervention trial would simultaneously treat these medical and psychological risk factors to determine if the prevalence of chronic postinjury pain can be reduced.

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Epigenetics of chronic pain after thoracic surgery

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Purpose of review

Chronic pain after surgery is a major public health problem and a major concern for perioperative physicians. Thoracic surgery presents a unique challenge, as thoracotomy is among the highest risk surgeries to develop persistent postsurgical pain. The purpose of this review is to discuss the relevance of research in pain epigenetics to patients with persistent pain after thoracic surgery.

Recent findings

Recent advances have linked chronic pain states to genetic and epigenetic changes. Progress in our understanding of chronic pain has highlighted the importance of immune modulation of pain. It is possible that epigenetic changes driving chronic pain occur in the perioperative setting via histone modification and DNA methylation.

Summary

The transition from acute to chronic pain after thoracic surgery may be mediated by epigenetics. Here, we discuss epigenetic modifications that have been discovered in animal models of chronic pain that may predispose patients to persistent neuropathic pain after thoracic surgery.

Keywords

chronic pain, epigenetics, thoracic surgery

INTRODUCTION

Chronic postsurgical pain is a major concern of surgeons and perioperative physicians as it represents the most common complication after thoracotomy [1,2]. Although nearly all patients have some degree of acute pain after surgery, 30–40% develop persistent chronic pain [3–6]. It is currently unclear what specific modifications occur in neural circuits responsible for nociception and what changes occur in biochemical pathways that increase the risk of developing persistent pain after surgery. Persistent postoperative pain after thoracic surgery is a key public health issue that needs to be addressed so that the prevention of acute and chronic pain may be better targeted [2]. Acute intercostal nerve injury and surgical tissue trauma may be the cause of chronic pain after thoracic surgery; however, there does not appear to be a strong correlation between presence of nerve injury and the development of chronic postsurgical pain [2,7].

There appear to be several important risk factors for developing persistent pain after surgery. One important predictor of persistent postoperative pain is presence of severe postoperative acute pain [8,9]. Interestingly, based on recent data, the presence of preoperative chronic pain does not necessarily predict severe postoperative pain [10]. Early

postoperative neuropathic pain is a risk factor [8]. Preoperative anxiety is an important risk factor for developing persistent postsurgical pain and may be a risk factor that should be optimized prior to surgery [3,11]. Female sex and increasing age also appear to be risk factors in developing chronic postoperative pain [3].

One important question that needs to be addressed is whether there is a difference in the incidence of chronic pain after video-assisted thoracoscopic surgery (VATS) or thoracotomy. It appears that the risk of developing chronic neuropathic pain after surgery is not related to mode of thoracic surgery whether VATS or thoracotomy [8,12]. Therefore, all patients presenting for thoracic surgery are

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KEY POINTS

- Chronic pain after thoracic surgery is a major public health concern.
- Epigenetic modifications may have a role in the development of chronic pain after surgery.
- Preclinical studies suggest that targeting epigenetic mechanisms may prevent the development of chronic pain after nerve injury.

at high risk of developing significant long-term morbidity from pain after surgery.

Recent advances in chronic pain medicine have linked innate immunity to the development and maintenance of chronic pain syndromes [13[■]]. Because of acute local and systemic inflammation that occurs after surgery, it is possible that the inflammatory environment postoperatively contributes to the generation of long-term chronic pain. In support of this hypothesis, patients undergoing lung transplantation surgery requiring perioperative and postoperative immune suppression have a lower incidence of developing chronic pain [14].

The root cause of the development of chronic pain after surgery, considering the aforementioned risk factors, is likely multifactorial with influences from both genetics (nature) and the environment (nurture). Epigenetics is a means by which the environment can alter the expression of genes [15]. The surgical stimulus that incites chronic pain may change and alter gene expression by epigenetic mechanisms. In this review, we will discuss the interplay between the environment and gene expression and focus on the key epigenetic targets as they relate to the development of chronic pain. There is promise from preclinical animal models in blocking epigenetic modification during acute pain that may prevent the progression to chronic post-surgical pain [16,17[■],18].

ENVIRONMENT, GENETICS AND PAIN

The role of genetics in pain phenotypes is undeniable. Genetic polymorphisms in a single gene can have such a profound effect on the sensation of pain that they can render individuals either insensate to pain or exquisitely sensitive to nonpainful stimuli. The gene SCN9A encodes a sodium channel (NaV1.7), and if mutated can cause either a gain of function resulting in erythralgia or loss of function resulting in the inability to sense pain [19–22]. The authors of this remarkable finding call the inability to sense pain as a result of a polymorphic

SCN9A gene ‘channelopathy-associated insensitivity to pain’ [20]. One patient cited in this manuscript, was found street performing including walking barefoot over hot coals and placing sharp knives through his skin [20]. This study demonstrates the key role of genetics in nociception.

In addition to purely genetic causes of altered nociception, environmental triggers of chronic pain may predispose to central sensitization. The role of environment in disease is often examined using twin studies. The question of whether identical twins both have an equivalent predisposition to the chronic pain remains incompletely answered; however, there are significant environmental factors, such as smoking, that correlate with chronic pain. These environmental factors possibly act through epigenetic mechanisms [23[■],24]. The remarkable plasticity of gene expression influenced by the environment may account for the variability in susceptibility for chronic pain among individuals sharing the same genetic predisposition. The aim of future studies should be on elucidating the modifiable environmental factors that may reduce the risk of developing chronic pain after surgery.

PAIN EPIGENETICS

Epigenetic mechanisms involve changing the likelihood of gene expression by altering the chemical or physical structure of DNA [15]. Structural and chemical changes in DNA may be influenced by experience (acute pain and perioperative inflammation) potentially predisposing patients to chronic pain after nerve injury because of surgical insult [18,25,26[■]]. Epigenetic modifications are an attractive target for therapeutics to block the development of chronic pain after surgery, as they are remarkably reversible and heavily influenced by environment.

One demonstration of how environmental influences can induce changes in gene expression through epigenetic modification came from the honeybee. It was discovered that feeding genetically identical larvae royal jelly beyond 3 days induces differential methylation in a gene that encodes a DNA methyltransferase (DNMT3) important in global gene expression [27]. The decrease in methylation of this gene, which occurs via exposure to the royal jelly beyond 3 days, results in the generation of a queen phenotype [27,28]. Remarkably demonstrated in the bee, feeding a nutritionally unique diet can cause epigenetic modification that profoundly influences phenotype. In contrast to the royal jelly, the inflammatory, stressful and traumatic nature of necessary surgical intervention may induce deleterious methylation changes that

result in a predisposition to develop a chronic pain after surgery. Possible epigenetic changes that occur in the perioperative period could be the driving force behind the development of persistent post-operative pain [18,29].

Epigenetic DNA changes occur via two major mechanisms: histone modification and DNA methylation [15]. The data relating to chronic pain via these two major mechanisms will be reviewed here.

HISTONE MODIFICATIONS AND THEIR ROLE IN THE EPIGENETICS OF PAIN

Scaffold proteins encircled by loops of DNA in the nucleosome are called histones. Histone proteins have protruding N-terminal tails that contain positively charged lysine residues that are sites for acetylation [30]. Histone acetylases (HAT) and deacetylases (HDAC) transfer or remove an acetyl group (from acetyl CoA) to and from positively charged lysine residues on histone tails that neutralize the charge. The positive charge on histone lysine residues attracts negatively charged DNA. Neutralizing the charge with an acetyl group weakens the binding of DNA to its histone scaffold [30]. Dissociation of DNA from histones via HATs relatively increases gene expression whereas HDAC exposes the positively charged lysine residues enhancing DNA attraction to histones and decreasing the likelihood of transcription. In addition to acetylation, histones may also be modified by methylation, phosphorylation, deamination and other mechanisms [30].

It has been shown that expression of HDAC is downregulated following spared nerve injury in animal models of neuropathic pain [31[■]]. This indicates (theoretically) that global gene expression is facilitated by HDAC in neuropathic pain models. In addition to this finding, a molecular mechanism for the suppression of *gad2*, a gene that encodes glutamic acid decarboxylase 65 important in pre-synaptic γ -aminobutyric acid (GABA) synthesis, involves HDAC [32]. In this study, Zhang *et al.* [32] demonstrated that using HDAC inhibitors and inducing a state of hyperacetylation caused analgesia in animals with inflammatory pain by increasing GAD65 activity. This exciting study suggests an epigenetic approach to treat and prevent chronic pain may prove successful.

HDAC inhibitors hold promise as an epigenetic treatment for the prevention of chronic pain in animal models. Given as an intrathecal infusion prior to nerve injury in rats, HDAC inhibitors cause reduced hypersensitivity relative to untreated controls [17[■]]. This effect was not present in animals treated with HDAC inhibitors at the same time as

nerve injury. This suggests that histone deacetylation during nerve injury is responsible for mechanical hypersensitivity and is reversible with HDAC inhibitor pretreatment. This preclinical study suggests promise in preventing nerve injury related histone modifications with HDAC inhibitors [17[■]]. This is an attractive approach when the risk of nerve injury and persistent postsurgical pain is high as in patients undergoing thoracic surgery.

Another recent study showed that expression of metabotropic glutamate receptor 2 (mGlu2) is regulated by histone acetylation in the dorsal root ganglion and mediates pain phenotype [33]. In order to achieve full transcriptional activity at the mGlu2 gene, deacetylation at the gene encoding nuclear factor- κ B transcription factor p65/RelA must occur. It has been recently demonstrated that the treatment with histone deacetylase inhibitors causes analgesia and also increased expression of mGlu2 in the DRG without affecting other metabotropic glutamate receptors [33]. This is a key finding linking histone acetylation to nociceptive phenotype. This exciting preclinical discovery suggests that therapeutic targets for treating and preventing chronic pain may be histone deacetylase inhibitors or mGlu2 agonists.

There is a strong link between chronic pain and inflammatory reaction emerging in the literature [13[■]]. There are many pathways of interaction between the nervous system and innate immunity. One of those pathways has a possible epigenetic linkage [34[■]]. Mice treated with partial sciatic nerve ligation to induce chronic pain had an increase in histone H3 acetylation in the promoter regions of the genes encoding macrophage inflammatory protein 2 (MIP-2) and C-X-C chemokine receptor 2 (CXCR2). This increased the recruitment and infiltration of inflammatory cells [via increased signaling between the mouse homolog of IL-8 (MIP-2) and its receptor CXCR2] [34[■]]. When treated with an inhibitor for HAT (preventing histone hyperacetylation), mice had a reduction in neuroinflammation and pain. This suggests a role for epigenetic modification in pain via modulating neuroinflammation [34[■]]. This also supports the growing link between chronic pain and inflammation.

DNA METHYLATION IN PAIN EPIGENETICS

DNA methylation is an epigenetic process by which cytosine residues are methylated by DNMTs in the CG dinucleotide sequence [35,36]. The degree of DNA methylation is inversely related to gene expression as the bulky methyl groups interfere with gene transcription [37]. It has emerged that covalent methylation of DNA cytosine residues is an

important mechanism that regulates chronic pain states and may be another potential epigenetic target to treat and prevent chronic pain [37].

In a recent study by Qi *et al.*, it was shown that the cystathionine- β -synthase (CBS) expression is epigenetically regulated via DNA methylation in the rat. CBS synthesizes hydrogen sulfide, which is involved in nociceptive signaling. In this study, mice exposed to complete Freund's adjuvant (CFA) had increased mechanical hypersensitivity and elevated CBS expression was found in the dorsal root ganglion [38[■]]. Using methylation-specific PCR and bisulfite sequencing, it was found that CFA-treated animals had less cytosine methylation in the *cbs* gene relative to control. This suggests that DNA methylation can influence the development of mechanical hypersensitivity in an inflammatory pain model. This result further suggests that DNA methylation may be a potential target for drug development in the treatment and/or prevention of chronic pain [38[■]].

In another study in rats, inhibiting DNA methylation with intrathecal 5-azacytidine attenuates the development of thermal hyperalgesia and mechanical allodynia in animals treated with a chronic constriction injury [39]. This suggests that blocking DNMT with 5-azacytidine reduces pain behavior in rats with induced nerve injury. Based on this preclinical study, DNMT inhibitors appear to be another promising epigenetically active therapy to prevent chronic pain after surgery [39].

There appears to be a significant supraspinal component to chronic pain and persistent postsurgical pain. Exciting work in mice has demonstrated that chronic pain is associated with global DNA methylation changes in the prefrontal cortex [26[■]]. It is well known that the prefrontal cortex is important in modulating chronic pain as well as facilitating depression and anxiety. It was shown that after spared nerve injury, mice had significantly reduced global methylation in the prefrontal cortex and the amygdala using a luminometric methylation assay. Furthermore, this group showed a strong correlation between severity of the behavior and percentage of global methylation, strengthening the link between nerve injury and induced methylation changes in the cortex. This study reinforces that nerve injury after surgery may induce DNA methylation changes that, if inhibited, may prevent the development and progression to persistent pain after surgery.

CONCLUSION

This review is focused on pain epigenetics as a potential target for preventing persistent postoperative

pain. Epigenetics is an emerging field in pain medicine as primary evidence is accumulating in preclinical animal models that epigenetic changes occur in chronic pain phenotypes. The interaction between the environment and genes via epigenetics is one that is important in the perioperative setting. Thoracic surgery patients undergoing VATS or thoracotomy have a high risk of developing persistent postsurgical pain. It is possible that surgical trauma and nerve injury triggers a cascade of epigenetic changes that results in altered gene expression and increased risk for developing chronic pain. Altering epigenetic processes with inhibitors of histone modification or DNA methylation may be an attractive target for preventing changes that occur in the perioperative period.

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Conflicts of interest

There are no conflicts of interest.

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ACUTE & PERIOPERATIVE PAIN SECTION

Original Research Articles

Epigenetics and the Transition from Acute to Chronic Pain

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Abstract

Objective. The objective of this study was to review the epigenetic modifications involved in the transition from acute to chronic pain and to identify potential targets for the development of novel, individualized pain therapeutics.

Background. Epigenetics is the study of heritable modifications in gene expression and phenotype that do not require a change in genetic sequence to manifest their effects. Environmental toxins, medications, diet, and psychological stresses can alter epigenetic processes such as DNA methylation, histone acetylation, and RNA interference. As epigenetic modifications potentially play an important role in inflammatory cytokine metabolism, steroid responsiveness, and opioid sensitivity, they are likely key factors in the development of chronic pain. Although our knowledge of the human genetic code and disease-associated polymorphisms has grown significantly in the past decade, we have not

yet been able to elucidate the mechanisms that lead to the development of persistent pain after nerve injury or surgery.

Design. This is a focused literature review of epigenetic science and its relationship to chronic pain.

Results. Significant laboratory and clinical data support the notion that epigenetic modifications are affected by the environment and lead to differential gene expression. Similar to mechanisms involved in the development of cancer, neurodegenerative disease, and inflammatory disorders, the literature endorses an important potential role for epigenetics in chronic pain.

Conclusions. Epigenetic analysis may identify mechanisms critical to the development of chronic pain after injury, and may provide new pathways and target mechanisms for future drug development and individualized medicine.

Key Words. Epigenetics; Pain; DNA Methylation; Histone Deacetylase Inhibitors; RNA Interference

Introduction

In recent years, we have developed a better understanding of the cellular mechanisms that link inflammation, peripheral sensitization, and pain [1]. In addition, we have learned more about the human genetic code [2] and mutations (particularly single nucleotide polymorphisms [SNPs] and copy number variations) that are associated with specific chronic pain syndromes [3,4]. These physiologic and genetic advances, however, do not fully explain why one patient develops chronic pain following an injury, and another patient does not. Despite recent improvements in techniques for acute pain management, 30–50% of patients still develop chronic pain following surgeries such as amputation, thoracotomy, hernia repair, and mastectomy [5].

It is also notable that monozygotic twins may exhibit significantly different inflammatory and chronic pain phenotypes [6–8], indicating that the etiological basis of these

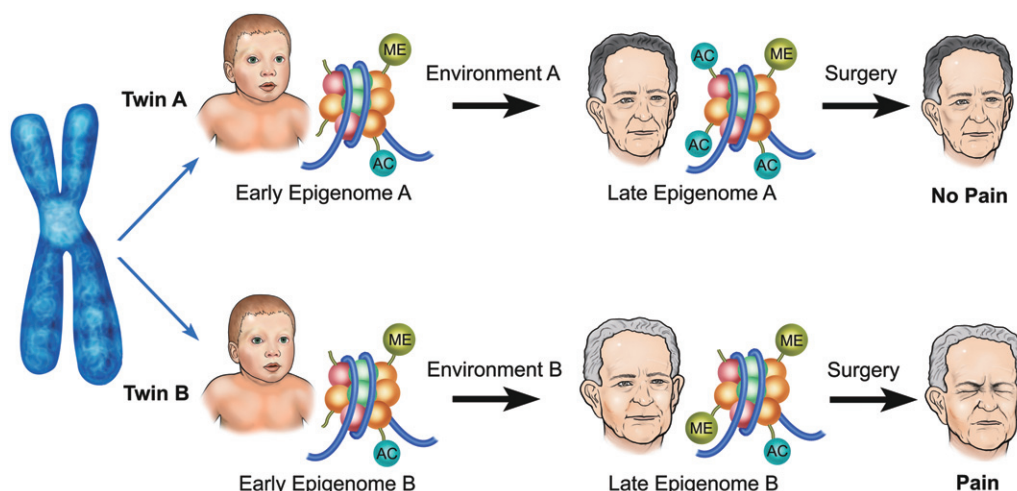


Figure 1 Epigenome and chronic pain. Twin A and Twin B demonstrate similar “epigenomes” at birth with few (if any) differences in methylation and acetylation patterns. Environmental factors throughout development affect histone acetylation patterns and cytosine methylation patterns, resulting in phenotypic differences by adulthood. With surgery or nerve injury, these epigenetic differences may result in differing risks of chronic pain.

disorders is not due simply to differences in genetic sequence. We now appreciate that response to injury is determined by complex interactions between the genome and the environment. These alterations might well be epigenetic in nature, i.e., heritable modifications that are not intrinsic to the genetic code, but that affect gene expression in a tissue-specific manner, resulting in an observable phenotype (Figure 1) [9].

Epigenetic processes are responsible for cellular differentiation during embryogenesis and are critical for normal development [10]. These processes also play an important role in memory formation, as correlations between hippocampal activity, DNA methylation, and histone phosphorylation in the brain have been found [11,12]. The spinal cord sensitization seen in painful conditions shares common mechanisms with the neural plasticity of memory formation [13], and it is likely that similar epigenetic mechanisms regulate both of these neural processes.

Multiple examples of the importance of epigenetic influences in development are found throughout nature. One of the best-described cases of environmental influence on gene expression involves the control of bee development by ingesting royal jelly. This nutritive substance induces changes in juvenile bee DNA methylation patterns and leads to development of the bee’s phenotype to become a queen rather than a worker [14]. The concepts of epigenetic heritability and stability have also been described in plants [15] and mammals [16]. For instance, high-fat diets fed to paternal rats induce functional changes in β -islet cells of female offspring [16]. Similar modifications in DNA methylation were noted in the fathers and

offspring, suggesting the nongenetic heritability of this metabolic disorder.

Nondevelopmental epigenetic modifications are also triggered by environment, nutrition, and stress [17–19], and may play a role in the onset of chronic pain following nerve injury [20,21]. We have long appreciated the importance of the psychosocial environment to the incidence and severity of chronic pain [22–27], and mounting evidence suggests that epigenetic mechanisms supply the link between disease expression and environment [18,28]. Nongenetic factors are important in the development of cancer [29,30], neurologic disorders [31], and painful disorders such as bladder pain syndromes [7], myofascial pain [32], and temporomandibular joint pain [8]. Twin disease models of neurodegenerative conditions [33], inflammatory periodontal disease [34], and autoimmune disease [35] demonstrate variable disease expression depending on the DNA methylation pattern [6].

Environmental factors alter gene expression and phenotype for painful disorders by inducing epigenetic modifications such as histone acetylation, DNA methylation, and RNA interference (RNAi) [36–38]. Following injury, expression of transcription factors such as nuclear factor-kappa B (NF- κ B) is increased [39], sodium channels in the injured axon are upregulated [40], μ -opioid receptors in the dorsal root ganglion are downregulated [41,42], substance P expression is altered [43], and the dorsal horn of the spinal cord is structurally reorganized through axonal sprouting [44]. As with DNA variation, epigenetic modifications may be inherited and may be propagated over multiple cell divisions; however, they are flexible enough to respond to

modifying influences. This concept may in part explain how we interact with our environment at the (epi)genomic level, and is potentially of great importance in understanding the relationship between gene expression and complex diseases such as chronic pain.

Genetics, Epigenetics, and Pain

Over the past several decades, much has been written about the association of genetic polymorphisms and the development of chronic pain [45,46]. It was believed that, through knowledge of genetic variation, we could develop the foundation for individualized medicine that optimizes therapy for each patient based on one's specific genetic sequence [47]. Expectations for personalized medicine were high after completion of the human genome project [2], but thus far, our ability to use the genetic code to prevent or improve chronic pain has been somewhat limited [48]. It is the heretofore unquantifiable environmental effect that has been one of the limitations of genetic studies [45].

Multiple candidate gene association studies have been used for the investigation of pain, but have been limited by their focus on genomic regions where the pathophysiology is thought to be reasonably well understood. They are not designed to analyze painful conditions that result from interactions of multiple genes [49]. A few candidate gene polymorphisms have been linked to pain susceptibility, including catechol-O-methyltransferase (COMT). This gene modulates nociceptive and inflammatory pain and has been linked to temporomandibular joint pain syndromes [50]. Even studies of COMT, however, have demonstrated inconsistencies. Some investigators have found an association between a COMT SNP val158met [4,50] with increasing pain responses, while others failed to replicate these findings [51,52].

The SCN9A gene has also been studied as a marker for pain sensitivity. Mutations in this gene, which codes for the alpha-subunit of a voltage-gated sodium channel (Na_v1.7), are known to result in alterations of pain perception [53], and have been noted in rare pain disorders such as erythromelalgia and paroxysmal extreme pain disorder [54,55]. SCN9A polymorphisms have also been described in individuals who are insensitive to pain [3,56]. Although the implications of the SCN9A gene polymorphism are clear, clinical applications of this knowledge remain limited [47].

Genome-wide association studies (GWAS) have been used in an attempt to overcome some of the limitations of candidate gene analysis. These studies tell us where the genetic variation exists, but do not always fully explain the underlying biology. Furthermore, although GWAS have identified thousands of genetic variations in complex diseases, most of the variants confer only a modest risk with an odds ratio for disease of <1.5. These genetic variants, therefore, account for only a small fraction of the population attributable risk for heritable complex traits [57,58], implying a strong nongenetic predisposition to disease.

GWAS directed toward painful conditions remain limited in number [45].

Specific Epigenetic Modifications

Histone Modifications

Histones octamers and their surrounding DNA form a nucleosome, the fundamental building block of chromatin (Figure 2A). The N-terminal histone tails may be modified by more than 100 different posttranslational processes including acetylation, phosphorylation, and methylation (Figure 2B). Most of the histone complex is inaccessible, but the N-terminal tail protrudes from the nucleosome and is therefore subject to additions that change the three-dimensional chromatin structure and subsequent gene expression [59,60]. One of the more common modifications involves acetylation. Histone acetyl transferases add acetyl groups, altering the histone protein structure. This change prevents the chromatin from becoming more compact, allowing transcription factors to bind more easily. This state of increased acetylation and "permissive chromatin" generally increases transcription activity and RNA production from that genetic sequence, especially when located in gene promoter regions [61,62]. Conversely, histone deacetylases (HDACs) remove acetyl groups from histones, generally suppressing gene expression. In concert, these activities serve important regulatory functions.

DNA Methylation

Another ubiquitous epigenetic modification involves methylation of DNA cytosine nucleotides. In this process, DNA methyltransferase enzymes (DNMT1, DNMT3A, and DNMT3B) add a methyl group to the 5-carbon of the cytosine pyrimidine ring, converting it to 5-methylcytosine. This methylation generally silences gene expression either by preventing the binding of transcription factors [63,64], or by attracting methylated DNA-binding proteins such as MeCP2 that themselves repress transcription (Figure 2C) [65,66]. The methylation process is vital for normal embryonic development and growth [67], and these methylation patterns are propagated during cell division.

The degree of cytosine methylation tends to mirror the degree of tissue specialization. For instance, DNA in neurologic tissue is highly methylated, while sperm DNA is relatively unmethylated [68]. More recent research has focused on the regulatory importance of cytosine methylation in promoter regions where methylation may silence a previously active gene sequence in the process of tissue specialization [69]. In addition to the cytosine nucleotides dispersed throughout the genome, there are regions particularly rich in cytosine-phosphate-guanine (CpG) linear sequences, described as "CpG islands" [70]. These "CpG islands" are found in promoter regions or first exons of approximately 60% of human genes, and are often unmethylated during development, allowing a transcriptionally active state [71]. Although promoter site methylation may

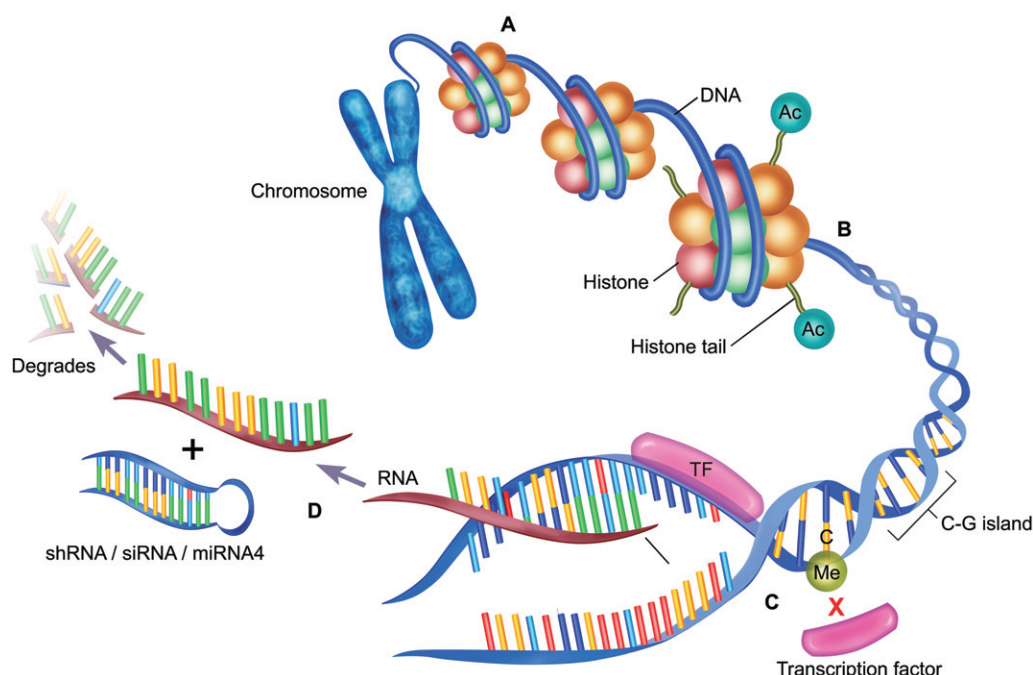


Figure 2 Epigenetic mechanisms. (A) DNA wraps around histone octamers to form a nucleosome, the fundamental building block of chromatin. (B) Histone proteins may be modified through several processes, including acetylation. The addition of an acetyl group to histone tails generally opens the chromatin structure and facilitates transcription factor binding, enhancing gene expression. (C) Methylation of cytosine nucleotides in C-G rich sequences (“CG islands”) prevents the binding of transcription factors and generally silences gene expression. These CG islands are often found near promoter regions and serve a significant role in gene regulation. (D) Posttranscriptional regulatory mechanisms include short hairpin RNA (shRNA), small interfering RNA (siRNA), and micro RNA (miRNA) that bind RNA and induce their degradation.

silence gene expression during development, genes may still be reactivated even in specialized neurologic tissues [72,73]. This potentially modifiable plasticity of neural tissue methylation may hold promise for reversing the neurologic molecular remodeling that occurs during the transition from acute to chronic pain.

Several disease states, including cancer, schizophrenia, and opioid addiction, are associated with DNA methylation abnormalities [30,74–76]. In cancer, these altered methylation patterns may lead to tumor growth by down-regulating tumor suppressor genes [30]. Methylated gene domains demonstrate not only stability, but also heritability [70]. The epigenetic influence across generations is demonstrated in rodent studies in which spermatogenesis is suppressed, and methylation patterns are altered for several generations after using the antiandrogenic compound vinclozolin during embryonic development [77].

Noncoding RNA

Gene expression can also be controlled by RNAi that involves endogenous molecules such as small interfering RNA (siRNA), microRNA (miRNA), and short hairpin RNA

(shRNA). These small noncoding RNA molecules can silence gene expression by binding to mRNA and inducing subsequent degradation of the direct gene product (Figure 2D) [78]. These molecules can self-propagate through cell division and epigenetically transmit regulatory information across generations [79]. Interfering RNAs carry great therapeutic promise and have been used in animal trials for chronic neuropathic pain [80] and neurodegenerative disease [81], as well as in human clinical trials for cancer [82].

Our understanding of epigenetic processes has increased dramatically over the past decade. Efforts are currently underway, through such groups as the International Human Epigenome Consortium, to sequence and create maps of cell-specific DNA methylation and histone modifications [83].

Techniques of Epigenetic Analysis

There are many challenges in defining the specific epigenetic changes that lead to a particular disease state. Many earlier epigenomic studies have been limited by either inadequate genome survey or small sample size, and the

relationship in many diseases between phenotypic expression and epigenomic variation remains unclear [84]. It is unlikely that single gene epigenetic modification will explain the complex pain phenotypes seen after injury or surgery. Epigenome-wide association studies have been proposed as a possible solution to improve our understanding of the links between disease state and epigenetic modifications. Comprehensive epigenomic maps are currently being developed with promising future applications [84].

Another challenge with epigenetic studies and disease variation is the need for enhanced comprehension of the distinction between cause and consequence [84]. To fully understand if a particular biomarker represents the cause of a disease or the effect from a disease, we will need to perform analyses at multiple time points before and after the development of a disease. This initiative has already begun with the establishment of the U.S. National Institutes of Health Roadmap Epigenomics Mapping Consortium [85].

Regardless of the relationship between biomarkers and causation, however, epigenetic modifications throughout the course of a chronic disease can be used as biomarkers. In particular, DNA methylation is well suited as a potential predictive biomarker secondary to its relative chemical stability. Reliable biomarkers are critical if we are to develop personalized epigenetic interventions. Candidate markers would need to be found in an accessible space (blood), but still reflect the neurobiological process occurring at the proximal tissue (spinal cord/brain). Whether the circulating leukocyte epigenome can report on more inaccessible tissues (such as central nervous system [CNS]) is uncertain, but there is growing evidence that methylation patterns tend to be similar between proximal tissue and more easily accessible circulating blood cells. For example, it was recently shown that the pattern of CpG island methylation in the promoter region of the prodynorphin gene in both human brain tissue collected postmortem and matched peripheral blood mononuclear cells is virtually identical [86].

The burgeoning field of epigenetics is using novel technologies to measure these heritable, yet modifiable, patterns of transcriptional regulation. DNA methylation is analyzed through bisulfite sequencing that allows the epigenetic information present in the form of cytosine methylation to be retained during amplification (Figure 3B). Traditional molecular analysis of specific gene loci relies on the ability to amplify the DNA of interest using cloning and polymerase chain reaction (PCR) techniques. If this amplification is done, however, without somehow immortalizing the methylation status of a particular cytosine, that information will be lost after the first PCR cycle. To solve this problem, unmethylated cytosines can be modified through the bisulfite reaction, deaminating them to uracil. Methylated cytosines, however, are not deaminated by bisulfite, remaining unchanged during subsequent amplification. Probes can then be designed to determine whether a specific promoter region has retained a particular cytosine (previously methylated) or whether this cytosine has been

converted to uracil (previously unmethylated). The methylation status of the promoter can then be determined using the cytosine/uracil ratio.

Histone protein modifications have also been studied since 1988 through a process of chromatin immunoprecipitation (ChIP) (Figure 3A) [87]. This process involves fragmentation of the chromatin and immunoprecipitation using an antibody to the protein or modification of interest. For example, an antibody to a specific acetylation site on histone H3 is used to precipitate all DNA associated with that particular acetylated histone. Following immunoprecipitation, the DNA fragments are then typically identified through microarray hybridization. More recently, “next generation sequencing” (NGS) technologies have been combined with ChIP, providing a high resolution, genome-wide analysis of histone modification. Whereas microarray techniques analyze regions of the genome previously identified, NGS carries the possibility of capturing all the DNA fragments isolated by immunoprecipitation [71]. These NGS technologies will continue to expand our understanding of epigenetic changes and the chromatin regulatory state throughout the genome.

The Role of Epigenetic Modification in the Transition from Acute to Chronic Pain

Prevention of chronic pain after injury has been the focus of numerous previous trials involving interventions such as multimodal analgesics and catheter-based local anesthetic infusions [88–90]. Although these techniques are successful in reducing the burden of acute pain [91], they have not succeeded in dramatically reducing the incidence of chronic post-injury or post-surgical pain [92–94]. The shortcomings of our preventive strategies are most pronounced following surgeries that have a higher risk for developing chronic pain such as amputation, thoracotomy, hernia repair, coronary artery bypass, and mastectomy [5,95,96].

Our therapeutic limitations may be partially due to our inability to prevent the epigenetic changes that occur following injury and surgery. A patient’s gene expression profile changes rapidly in the post-injury period [97], with over 1,000 genes activated in the dorsal root ganglion alone after nerve injury [98]. There is significant evidence for epigenetic control of this gene activation in the transition from acute to chronic pain. First, immunologic response and inflammatory cytokine expression are under epigenetic control [99,100]. Second, glucocorticoid receptor (GR) function, which affects pain sensitivity, inflammation, and the development of autoimmune disease, is modulated both through posttranslational mechanisms and DNA methylation [101–103]. Third, genes such as glutamic acid decarboxylase 65 that code for pain regulatory enzymes in the CNS are known to be hypoacetylated and downregulated in inflammatory and nerve injury pain states [104]. Finally, epigenetic modifications are involved in opioid receptor regulation and function, with implications for endogenous pain modulation systems and pain severity [63,76].

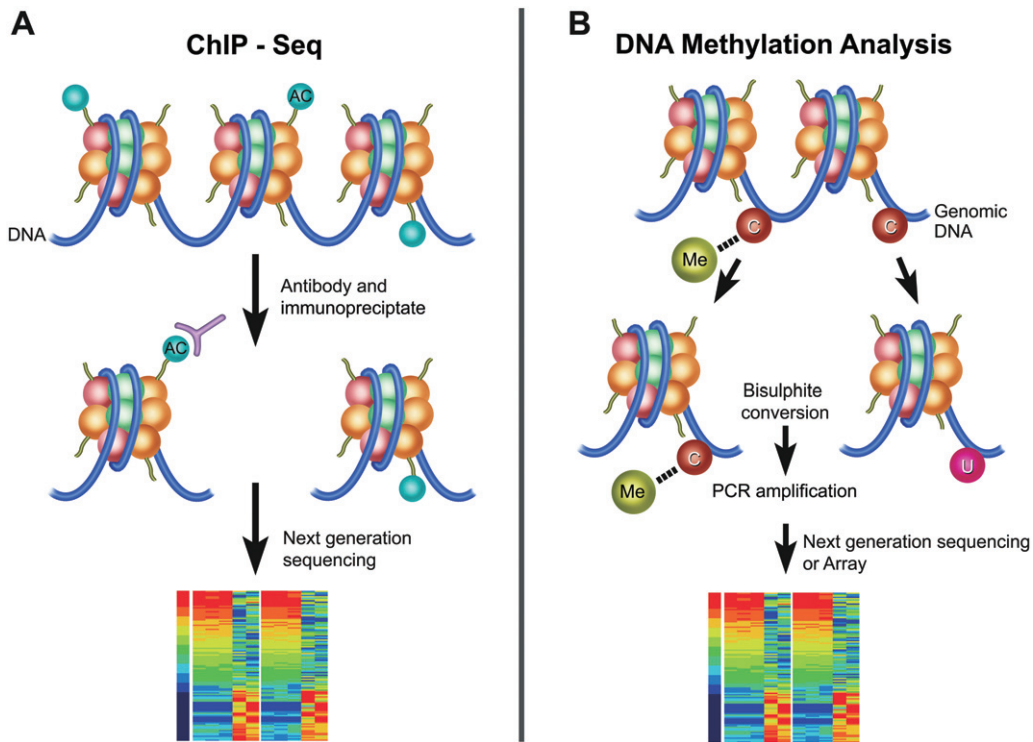


Figure 3 Laboratory techniques in epigenetics. (A) In ChIP-seq analysis, an antibody is used on chromatin to immunoprecipitate and select for acetylation and other histone modifications. The results may then be analyzed through several techniques including genome-wide next generation sequencing. In this manner, the histone acetylation patterns of a particular tissue may be determined. (B) The analysis of DNA methylation employs bisulfite sequencing to convert unmethylated cytosines to uracil. This process does not affect the methylated cytosines. The methylation patterns can be calculated by comparing the ratio of cytosine to uracil.

The important link between epigenetic regulation and pain is also supported by studies involving intervertebral disc degeneration and chronic low back pain. Tajerian et al. found that DNA methylation of an extracellular matrix protein, secreted protein, acidic, rich in cysteine, is linked to accelerated disc degeneration both in humans and in animal models of this disease [38]. The correlation between pain and epigenetics is additionally observed in a study of DNA methylation in human cancer where endothelin receptor type B (EDNRB) is heavily methylated and downregulated in painful squamous cell carcinoma (SCC) lesions [105]. The investigators noted similar findings in their mouse model of SCC, and were able to improve mechanical allodynia when EDNRB transcription was virally augmented [105]. These human and animal studies strongly support a role for gene methylation in regulating the pain experience.

Cytokines

Injury and autoimmune disease are characterized by excessive cytokine production, and anti-cytokine thera-

pies have been successfully used to treat painful conditions such as ankylosing spondylitis [106,107] and neuropathy [108,109]. The link between cytokine expression and pain is supported by the demonstration of T-cell infiltration and inflammatory interleukin (IL) release in animal models of neuropathic pain [110]. Furthermore, interventions that modify the immune response to injury also reduce pain. Such modifications include depletion of mast cells [111], reduction of peripheral macrophages using clodronate [112], and impairment of complement activation and neutrophil chemotaxis [113].

One of the inflammatory master switches, nuclear factor- κ B (NF- κ B), induces multiple cytokines [114] and cyclooxygenase [115]. NF- κ B is epigenetically regulated by acetylation and remodeling of chromatin [114,116,117]. When activated, this transcription factor demethylates and induces cytokines such as Tumor necrosis factor- α (TNF- α), IL-1, IL-2, and IL-6 [118,119]. Activation of NF- κ B is associated with autoimmune and neurodegenerative disease [120]. Conversely, inhibition of NF- κ B reduces pain behavior after peripheral nerve injury [121].

The link between epigenetically induced cytokine production and pain intensity has been noted in multiple disease models such as migraine headache [122], diabetes [114], and osteoarthritis [99]. In osteoarthritis, DNA demethylation at specific CpG sites in human chondrocytes produces aberrant expression of inflammatory cytokines (IL-1 β) and metalloproteinases [99]. Thus, cytokine-induced painful joint damage appears to be epigenetically modulated.

GRs

Glucocorticoids are important endogenous regulators that appear to protect against excessive inflammatory response following injury. Stress-induced glucocorticoid production suppresses immune cell release of IL-6, TNF- α , and other inflammatory cytokines [123]. Exogenous glucocorticoids also have potent anti-inflammatory actions and are used extensively in the treatment of autoimmune disease and painful conditions. However, not all patients respond equally to their clinical effects, and it is believed that glucocorticoid resistance is a likely mechanism in the development of autoimmune disease and chronic pain [124].

The GR is controlled by a system of complex regulatory mechanisms, and clinical response to glucocorticoids correlates with the number of intracellular GRs [125]. Normally, individuals demonstrate variable GR promoter methylation [103] and variable response to glucocorticoid therapy [126]. Diverse methylation patterns are believed to lead to the use of alternative promoter sites and subsequent alteration in GR sensitivity [103].

GR expression is also modified by maternal care, grooming, diet [127,128], and early-life stresses [129,130]. Human studies have demonstrated epigenetic alterations in GRs of patients who previously suffered abuse [131]. The style of maternal care appears to specifically affect methylation patterns of exon 1₇ of the GR promoter, epigenetically linking receptor function and early-life experience [132]. Abnormalities in GR-mediated immune cell function may lead to the development of inflammatory adult phenotypes [133] and autoimmune disorders such as rheumatoid arthritis [101,134]. GR dysfunction may also play a role in fatigue, chronic pain states, and fibromyalgia [102,135]. These maternally influenced expression patterns, however, are not necessarily permanent and have been reversed in cross-fostering parent studies [136]. The GR appears to provide a potential link between injury, environmental stresses, and the severity of chronic pain.

Opioid Receptors

Both demethylating agents and HDAC inhibitors increase expression of the μ -opioid receptor [137], indicating that the endogenous opioid system is under significant epigenetic control. Consistent with these laboratory findings, increased CpG methylation has been noted in the promoter regions of the μ -opioid receptors of heroin users,

consistent with receptor downregulation [76]. Likewise, DNA methylation of the proenkephalin gene promoter inhibits transcription and gene expression of this opioid peptide [63].

Beyond the direct role of methylation in the regulation of opioid peptide expression, spinal opioid receptor activity also appears to be partially modulated by central GRs [138]. This association is of particular importance given the synergy between the increased central expression of GR following peripheral nerve injury [139] and direct epigenetic manipulation of the endogenous opioid system [63,137]. The interaction between modifications of the GR and the opioid receptor demonstrates the complex role that epigenetic alterations play in controlling the inflammatory and pain-modulating pathways.

“Epigenetic Intervention” to Prevent Chronic Pain

Genetic studies have taught us that variability in pain sensitivity results from multiple genetic and environmental factors. Environmental influences upon pain severity have been previously described and linked to early-life stress [47,140–143]. Although precise mechanisms have yet to be elucidated, epigenetic modifications are increasingly appreciated as a likely factor in this linkage [36,104,122].

Our need for targeted therapies has never been greater. Multiple analgesic drugs are now in use; however, most of these share a common function with opioids or anti-inflammatory medications. These medications have improved symptoms in some patients, but have created the additional morbidities of systemic toxicity, opioid tolerance, and addiction. Our options for safe and effective treatments for chronic pain remain limited with few recent “breakthroughs.”

Since the sequencing of the human genome, there have been increasing calls for “personalized medicine” that tailors drug therapy to a patient’s pain phenotype [47,144]. Although such therapies have demonstrated some efficacy as cancer treatments [145–147], we have not yet had great success with targeted pain therapies. We will now review some of the potential targets for “personalized epigenetic intervention” (Table 1).

Intervention: HDAC Inhibition

Given the association between histone deacetylation and cancer, neurodegenerative disease, and pain, histone deacetylase inhibitors (HDACis) have been evaluated as therapeutic agents for these diseases [30,36,148]. Thus far, HDACis are primarily used in cancer therapy. In these patients, HDACis alter the balance of acetylation/deacetylation and activate genes that suppress tumor growth and invasion [30,149–152]. In neurodegenerative disease, HDACis have been evaluated secondary to their ability to induce neural growth and to improve memory [153]. HDACis have also demonstrated evidence for

Table 1 Epigenetically active drugs and their mechanisms

Epigenetics Mechanism	Drug	Action	Clinical Use	Comments
Histone deacetylase inhibitor	Valproic acid	Inhibits classes I and II HDAC	Seizures, pain	Effective for migraine prophylaxis
	Givinostat	Inhibits classes I and II HDAC	Juvenile idiopathic arthritis	Effective in human arthritis trial
	Tricostatin A (TSA)	Inhibits classes I and II HDAC	Laboratory only	Produces analgesia in animal models. Enhances μ -opioid receptor transcription
	Suberoylanilide hydroxamic acid (SAHA)	Inhibits classes I HDAC	Laboratory only	Produces analgesia in animal models
DNA methylation	Glucosamine	Prevents demethylation of IL-1 β gene promoter	Arthritis pain	Common clinical use; effect on IL-1 β reduces inflammatory cytokine production
	Valproic acid	Induces demethylation of reelin promoter	Seizures, pain	Reelin modulates NMDA function and pain processing
	L-methionine	Induces methylation at glucocorticoid receptor promoter gene	Dietary supplement	Alters experimental stress response; used as dietary supplement for arthritis
RNA interference	siRNA targeted to NMDA receptor subunits	Gene silencing of NR1 and NR2 subunits of NMDA	Experimental	Produces analgesia in animal models
	siRNA to P2X3	Gene silencing of P2X3	Experimental	Produces analgesia in animal models; no observed neurotoxicity with intrathecal use
	siRNA to TNF- α	Gene silencing of TNF- α	Experimental	Produces analgesia in animal models

analgesia in both inflammatory and neuropathic pain [151,154,155]. The clinical effect of many of these drugs is thought to be partially attributed to reduced production of inflammatory cytokines such as TNF- α and IL-1 β [156].

HDACis are organized into several different structural groups. Trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) are hydroxamate-based HDACis. TSA inhibits both class 1 (ubiquitously expressed) and class 2 (selectively expressed) HDACs, whereas SAHA exhibits greater selectivity for class 1 HDAC. TSA produces analgesia in animal models with an associated decrease in expression of transient receptor potential type-1 cation channel (TRPV1) and protein kinase C ϵ [157]. SAHA reduces the nociceptive response of animals during the second phase of the formalin test [154]. These drugs increase acetylation of the transcription factor p65/RelA, which enhances gene expression of the metabotropic glutamate receptors (mGlu2) in dorsal root ganglia neurons. Activation of these mGlu2 receptors inhibits primary afferent neurotransmitter release in the dorsal horn of the spinal cord and provides analgesia in animal models of neuropathic pain [158]. TSA also enhances μ -opioid receptor transcription [159], indicating partial HDAC modulation of the endogenous opioid system.

Another HDACi, Givinostat, has not only demonstrated evidence of analgesia in animal models, but also efficacy in a human trial for juvenile idiopathic arthritis. Although randomized studies have not yet been performed, its use for this autoimmune inflammatory disease is especially encouraging given its relative lack of systemic toxicity [160].

The most commonly used HDACi, valproic acid (VPA), is part of the aliphatic-based drug class that inhibits classes I and II HDACs [151,161], and is effective following systemic or intrathecal administration [162,163]. VPA is of particular interest because it has been successful with long-term clinical use [164]. Although it is now used predominantly to treat chronic painful conditions [163–165], its inhibition of HDAC and potential to prevent specific epigenetic alterations may lead to preemptive use in the acute setting. It is not yet clear whether VPA-induced analgesia results from HDAC inhibition or its ability to potentiate gamma amino butyric acid (GABA) in the CNS.

Although therapies based on HDAC inhibition have been effective in treating pain and oncologic disease, nonspecific HDACis such as TSA affect the regulation of multiple

genes, which increases the possibility of side effects with this therapy [166,167]. The success of future drug development will likely depend upon our ability to target specific subclasses of HDACs that selectively alter pain processing without the toxicities of nonselective agents. The importance of this selectivity concept has been demonstrated in a mouse model in which a full knockout of the HDAC4 gene (a class IIa HDAC) is lethal, whereas a conditional knockout of this gene provides analgesia [168]. Further investigations of HDAC subclass function are needed in order to identify novel drug targets.

Intervention: DNA Methylation

DNA methylation is another key epigenetic mechanism. Methylation patterns, although generally stable throughout the genome, are responsive to pharmacologic intervention. One common medication that appears to act through epigenetic mechanisms is glucosamine [169]. In arthritis models, it has been demonstrated that glucosamine prevents demethylation of the IL-1 β gene promoter, thereby decreasing expression of this cytokine. Decreased IL-1 β subsequently reduces NF- κ B expression and downstream inflammatory cytokine production [119,170].

In addition to its function as an HDAC inhibitor, VPA induces demethylation of multiple genes [171]. One of these important genes encodes for reelin, a glycoprotein synthesized by GABAergic neurons of the CNS [172,173]. Reelin modulates N-methyl-D-aspartate (NMDA) receptor function [174], and is important for sensory processing [175]. Mutations of this gene cause alterations in mechanical and thermal hypersensitivity [173], which indicates the potential significance of VPA regulation of reelin in the development of chronic pain.

L-methionine administration has also been tested as a potential drug for epigenetic intervention. This amino acid appears to increase methylation patterns of the GR gene, thereby altering the hypothalamic-pituitary-adrenal response to stress [176]. In addition, dietary methyl supplementation in an animal model improves the health and longevity of offspring [177]. Both of these findings suggest that nutritional status partially controls the activity of the GR and its role in inflammatory disease.

The combined action of pharmacologic DNA demethylation and HDAC inhibition increases activity at the proximal promoter site of the μ -opioid receptor gene, increasing μ -opioid receptor expression [137]. Carried out in concert, these processes may represent an important balance that allows less stable histone modifications to lead to more stable changes in DNA methylation, thus facilitating longer-term modifications in the endogenous opioid receptor system.

Intervention: RNAi

Epigenetic therapies based on RNAi also hold promise for preventing and treating chronic pain. These methods target specific disease pathways.

RNAi is an endogenous mechanism for gene silencing in plants [178] and mammals [179], and involves subgroups such as siRNA, miRNA, and shRNA. Given their ability to silence undesirable gene products in malignancy, these small RNA molecules have been used for cancer therapy [82]. They have also been shown to improve chronic neuropathic pain [80].

siRNA targeted for the NR2 subunit of NMDA receptors abolishes formalin-induced pain behavior in rats [180]. Likewise, injection of siRNA aimed at the NR1 subunit of the NMDA receptor alleviates experimentally induced allodynia in mice [181]. Successful RNAi studies have targeted TRPV1 channels [182], brain-derived neurotrophic factor [183], cytokines such as TNF- α [184], and pain-related cation channels (P2X₃) [80]. Importantly, direct intrathecal administration of siRNA targeting P2X₃ in animals has not demonstrated significant toxicity [80], indicating that this intervention may be applicable to humans in coming years.

Conclusions

The transition from acute to chronic pain is a complex process involving local inflammation and nociceptor activation that may resolve in some patients and may lead to the development of chronic pain in others. As we learn more about the various ways that injury and environment change gene expression, we can begin to elucidate disease mechanisms and gain insight into potential therapies. Epigenetic alterations such as DNA methylation, histone acetylation, and RNAi are necessary for normal tissue specialization and neurologic development. However, these same modifications play a significant role in the induction of the chronic pain phenotype following neurologic injury.

In contrast to the genetic determinism inherent in genomic studies, the field of epigenetics strives to understand the environmental control over gene expression. Such knowledge will open up opportunities for developing novel analgesics. Future personalized therapies will likely be based on epigenetic interventions that alter the transcriptional expression that occurs in chronic pain states. Given the strong mechanistic implications of epigenetic modifications in the development of chronic pain, and our current treatment limitations, we possess both the promise of epigenetic tools and the imperative to prevent the transition from acute to chronic pain.

Authors' Contribution

TB, TV, and AS conceived, wrote, and performed the final editing of this manuscript. Medical illustrations were created in collaboration with Stan Coffman from Media Solutions, Durham, NC. We also wish to thank Kathy Gage, BS, Duke University Department of Anesthesiology, for her editorial assistance in the preparation of this work.

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Causes and Prevention of Chronic Post-Surgical pain

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Abstract

Purpose of review: Surgical incision invariably causes some measure of nerve damage and inflammatory response that, in most cases, heals quickly without long-term negative consequence. However, a subset of these patients go on to develop lasting neuropathic pain that is difficult to treat and, in many cases, prevents the return to normal activities of life. It remains unknown why two patients with identical surgical interventions may go on to develop completely divergent pain phenotypes or no pain at all. Aggressive, early analgesic therapy has been shown to reduce the incidence of chronic post-surgical pain (CPSP), but no specific regional anesthetic technique or systemic pharmacologic therapy has been shown to prevent CPSP.

Recent Findings: Inflammation and glial cell activation have recently been shown to be just as important in the transition from normal acute pain to pathologic chronic pain as nerve injury itself and that central sensitization may not be solely due to repetitive nociceptive firing at the time of nerve injury. This has opened a number of new therapeutic possibilities for prevention of CPSP.

Summary: Here we discuss the causes of CPSP and current useful preventative strategies in the perioperative period. We also discuss future potential disease modifying treatments of CPSP.

Keywords:

Surgical Nerve Injury, Chronic Pain, Neuropathic Pain

The long-lasting, life-changing painful sequelae of nerve injury have been recognized for many years. S. Weir Mitchell, a neurologist who cared for amputees of the United States Civil War said:

*Perhaps few persons who are not physicians can realize the influence
which long-continued and unendurable pain may have on both mind
and body . . .*

He particularly noted the post-amputation burning pain syndrome, which he termed causalgia, to be “the most terrible of all tortures which a nerve may inflict.” [1]

Though the chronic pain conditions of many war-wounded have been described by physicians like Mitchell, Leriche, and Livingston, it was not until the 1980s that the medical community began to recognize the growing problem of chronic pain following surgery. [2, 3] Since then, a number of small studies have been conducted to examine the incidence and prevention of chronic post-surgical pain (CPSP), though few have been large, prospective, or randomized.

Each year in the United States, more than 40 million people undergo surgical procedures. Many of these are relatively non-invasive, including colonoscopy and cataract removal; however, large numbers of patients undergo major procedures that result in varying degrees of nerve damage with the potential for the subsequent development of CPSP. For example, 1.7 million people in the US are survivors of limb loss, and each year over 130,000 new amputees are added to that number.[4] It has been estimated that 50%-80% of these patients experience significant, long-term phantom or residual limb pain for months to years following surgery.[5] And 5%-10% of these patients develop severe, life-changing, chronic pain. By adding all the patients who undergo thoracotomy, caesarean section, herniorrhaphy, and breast surgery, the number of new chronic pain cases approaches half a million each year (Table 1). The estimated cost in lost productive time from chronic pain conditions like CPSP is over \$60 billion dollars annually.[6] The burdens of related substance abuse, depression, and complications of opioid treatment are more difficult to quantify but no less substantial.

Causes of Chronic Post-Surgical Pain

In 1983, Woolf published the first paper demonstrating the importance of central sensitization to the production of chronic post-injury pain.[16] Soon after, it was suggested that surgical incision causes inappropriate nerve firing, which leads to this sensitization.[2]

In addition to simple repetitive nociceptor firing, recent studies have revealed that the source of central sensitization can include perioperative noxious stimuli, surgical stimuli, and inflammatory mediators.[17] Important work during the past decade has identified inflammation and glial cell activation as generators of neuronal sensitization, [18] greatly expanding the possibilities for future therapeutics beyond simple prevention

of nociceptive signaling.

The Immune Response to Nerve Injury

Peripheral nerve injury is immediately followed by a robust inflammatory response. Macrophages localize to damaged nerve fibers. Macrophages and lymphocytes gather upstream in the dorsal root ganglion (DRG), and activate microglia and, eventually, astrocytes in the central nervous system (CNS).[18, 19] Growth factors released by Schwann cells at the site of injury sensitize nociceptors directly.[20] Distal nerve injury recruits macrophages proximally to the DRG where, in humans, the immune response continues months after the distal inflammation has resolved.[21] In the dorsal horn of the spinal cord, large numbers of microglia, which share a common myeloid lineage with peripheral macrophages, and circulating monocytes surround the terminals of the injured nerve fibers. In response to chemokines, such as fractalkine and CCL2,[22] and Toll-like receptor signaling.[23] These activated microglia lead to increases in levels of the pro-inflammatory cytokines IL-1 β , IL-6, and TNF α . These cytokines contribute to neuropathic pain symptoms, such as mechanical allodynia, by directly sensitizing dorsal horn neurons. This series of discoveries describes a completely different paradigm for chronic nociceptor sensitization and, potentially, an important new therapeutic target.

Risk Factors for Developing Chronic Post-Surgical Pain

While many patients who undergo surgical procedures develop chronic pain, many do not. Indeed, patients undergoing apparently identical surgical procedures often have divergent post-operative pain experiences. For example, one below-the-knee amputee may develop severe, unending, disabling phantom limb pain following surgery, while

another may be pain-free after the surgical incision has healed. This phenomenon has yet to be explained, though data on predisposing risk factors for pain development continue to be gathered.

Comorbid psychosocial factors such as uncontrolled perioperative anxiety,[24] post-traumatic stress disorder, and predisposition to catastrophizing[25] correlate with the development of CPSP. Lack of social support or an overly solicitous spouse has also been associated with worsened pain scores following amputation. A number of specific genetic polymorphisms have been linked to the incidence of CPSP, and these may lead to the discovery of pathways that control the transition from acute to chronic pain.

Severe preoperative and immediate postoperative pain is a strong predictor of the development of chronic pain following a variety of surgical procedures.[26-28] The reason for this association remains unclear but possible explanations include 1) structural CNS changes caused by the presence of severe preoperative pain, 2) an unknown genetic factor that predisposes some patients to both acute and chronic pain, and 3) anxiety and a tendency toward catastrophizing in patients who report more intense pain than those without these psychological traits.[29] The association between severe perioperative pain and the development of chronic pain, however, does provide at least one important avenue for preventing CPSP and has been the foundation for using regional anesthetic techniques perioperatively to block nociception and subsequent sensitization.

Prevention and Treatment

Regional and Neuraxial Analgesia

It is difficult to talk about treatment of chronic post-surgical pain without also discussing prevention strategies. Once entrenched, central sensitization leads to pain phenotypes

that are very difficult to reverse and extremely complicated to treat. Prevention, then, currently appears to be the most promising way to reduce the overall incidence of CPSP, though current strategies have not been as successful as hoped.

Early studies examining the incidence of CPSP after amputation in the setting of perioperative epidural analgesia resulted in some encouraging findings. A 1988 unblinded epidural analgesia study reported reduced phantom limb pain at 6 and 12 months in patients receiving epidural bupivacaine and morphine for 72 hours before amputation compared to controls.[30] This was followed by a case control study of 24 patients treated with epidural bupivacaine, clonidine, and morphine who showed an 8% incidence of phantom limb pain compared with 73% in a control group.[30] However, when confirmatory, randomized prospective studies were performed to confirm these findings, the results proved to be much different. Nikolajsen and colleagues randomized patients to groups receiving epidural analgesia for the entire perioperative period and patients receiving epidural analgesia postoperatively only.[31] At 12 months, phantom limb pain was present in 75% of the patients who received epidural analgesia throughout the perioperative period and in 69% of those receiving postoperative analgesia only. Karanikolas and colleagues in 2010 attempted to determine which of five analgesic regimens, including epidural analgesia in three of the five regimens, reduced chronic phantom limb pain after amputation. [32] This study concluded that epidural analgesia throughout the perioperative period was no different than patient controlled opioid analgesia throughout the perioperative period at reducing the prevalence of chronic phantom limb pain.

Similar results were found when studying peripheral nerve blockade as a method for preventing CPSP. In 1991, Fisher and Meller published an observational study that followed 11 patients who had undergone lower extremity amputation with intraoperative

placement of a perineural catheter.[33] None of these patients had developed phantom pain at 12 months. Unfortunately, as with epidural analgesia, when followed up with prospective, randomized studies, these exciting results were not reproduced. In 1996, Pinzur and colleagues[34] randomized amputation patients to receive bupivacaine or saline through an intraoperatively-placed catheter. No difference in the incidence of phantom limb or stump pain was reported.

Recently, Borghi and colleagues,[35] studied preoperative percutaneously placed peripheral nerve catheters in patients about to undergo amputation. A local anesthetic infusion was not administered preoperatively, but a postoperative infusion was continued for a median duration of 30 days. The incidence of phantom limb pain at 12 months was 16%, much lower than the background incidence of phantom limb pain noted in other studies. Though encouraging, this was not a randomized trial.

This section has focused mainly on regional anesthesia in CPSP after amputation, but the conclusions are applicable across multiple surgeries including thoracotomy, as neither epidural analgesia nor paravertebral nerve blockade has been proven to reduce the incidence of severe, chronic post-thoracotomy pain. Providers quickly learn, when treating patients with chronic pain, that there is no simple treatment, straightforward etiology, or uncomplicated prevention strategy. Therefore, it is unsurprising that simple blockade of nociceptive input has not yet been proven sufficient to prevent chronic sensitization. Regional anesthetic techniques, however, remain a vital part of the effort to reduce pain perioperatively, as perioperative pain severity has been strongly associated with the development of CPSP.

Systemic Analgesia

Regional anesthetic techniques have not proven to be sufficient to prevent chronic pain after surgery, thus, a multimodal approach to perioperative pain management remains important. Opioids continue to be the mainstay of acute pain control, but a number of other pharmacologic agents can act as adjuvants in the perioperative period.

NSAIDS and acetaminophen have an opioid-sparing effect and are efficacious in controlling acute pain following amputation and thoracotomy,[36, 37] but there is no evidence that these medications prevent chronic post-surgical pain.

The α_2 adrenergic agonist, clonidine, is often used to treat both acute and chronic neuropathic pain. Interestingly, clonidine has a role in regulating the levels of inflammatory cytokines following nerve injury; and in an animal model of sciatic nerve injury, clonidine reduces mechanical hypersensitivity.[38] Clonidine's ability to prevent CPSP is unknown, but its anti-inflammatory and anti-sensitizing effects make it a prime candidate for further study.

The NMDA receptor antagonist, ketamine, has been used successfully to treat acute postoperative pain, especially in chronic pain patients.[39, 40] Because activation of the NMDA receptor is required for the development of central sensitization, it was hoped that partial blockade of NMDA receptor activity by drugs like ketamine during the perioperative period would reduce the incidence of CPSP, but this has not been the case in subsequent studies.[41, 42]

Gabapentin and pregabalin are calcium channel blockers that reduce neurotransmitter release and neural sensitization. Gabapentinoids reduce opioid requirements as well as acute pain in the perioperative period.[43] When used without regional anesthesia, gabapentin reduces CPSP. However, in another study by

Nikolajsen and colleagues, amputation patients, most of whom also received epidural analgesia, were randomized to receive gabapentin or placebo after amputation.[44] No difference in the incidence of chronic phantom limb pain was found.

Future prevention modalities

While sufficient analgesia before, during, and after surgery is important for the prevention of chronic post-surgical pain, mere short-term blockade of nociception and control of symptoms have not been shown to eliminate this long-term problem. Knowledge of the pathophysiologic processes causing the transition from useful, short-lived acute pain to pathologic, destructive chronic pain has expanded significantly over the past decade, as the vital role of inflammatory mediators and glial cell activation in nociceptor sensitization has been revealed. Improved understanding of these, and other novel chronic pain mediating pathways creates the potential for disease modifying treatments rather than symptom-focused treatments. For example, a new class of circulating anti-inflammatory molecule, called resolvins, have recently been described and found to have endogenous analgesic effects.[45, 46] Resolvins are lipid mediators derived from omega-3 fatty acids that are produced in leukocytes and have been found to attenuate inflammatory pain by inhibiting neutrophil infiltration and pro-inflammatory cytokine expression. Also, Resolvin D2 when administered intrathecally in mice, is able to reverse the long-term potentiation created by tetanic stimulation of C-fibers in the sciatic nerve [46]. Other potential novel preventative treatments could target purinoreceptors, like P2X4 in microglia, whose activation causes dorsal horn neuron hyperexcitability [47, 48]. Treatment with growth factors such as glial cell line derived neurotrophic factor (GDNF) have been shown to reduce neuropathic pain development

in animal models of CPSP[49]. Blockade of growth factors like NGF appear to inhibit chronic nociceptor sensitization [50].

Conclusion

Current acute post-surgical pain treatment involves analgesia targeting opioid receptors, cyclooxygenase, nociceptor signaling and NMDA receptor signaling. This multimodal approach has transformed the patient experience of pain immediately following surgery, but has not substantially lessened the long-term disease burden of those unfortunate patients who undergo the often catastrophic transition to chronic post surgical pain. Continuing to improve the understanding of the mechanisms behind this transition is vital to expanding our current multimodal approach to include disease modifying preventative treatments for chronic post surgical pain.

- **Surgical procedures involving damage to large nerves, such as amputation and thoracotomy, are associated with up to an 80% incidence of chronic pain with up to a 10% of those severe and disabling.**
- **Our understanding of the variability of chronic pain development between patients with identical surgical procedures is limited.**
- **New information linking inflammation and glial cell activation to chronic neuropathic pain has opened the possibility of novel therapeutics.**
- **At this time, aggressive perioperative analgesia is the only known effective way to prevent CPSP.**

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Prevention of Chronic Pain After Surgical Nerve Injury: Amputation and Thoracotomy

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KEYWORDS

- Chronic pain • Surgical nerve injury • Amputation
- Thoracotomy • Neuropathic pain

ACUTE POSTSURGICAL PAIN

A surgical incision produces tissue damage, subsequent inflammation, and acute postoperative pain. Although most patients heal without long-term sequelae, procedures, such as amputation, thoracotomy, hernia surgery, coronary artery bypass, and mastectomy, impose a significant burden of persistent postsurgical pain.¹⁻³ However, amputation and thoracotomy represent two of the higher-risk procedures. These surgeries involve obligatory neurologic injury, often leading to a cascade of postinjury sensitization and chronic neuropathic pain.^{1,4}

Although amputation and thoracotomy have different indications and are performed using different techniques, they demonstrate a remarkable similarity both in the severity of acute postoperative pain and in the incidence of persistent postsurgical neuralgic pain.¹ Our ability to control incisional and inflammatory pain in the immediate postoperative period has improved with the combined use of local anesthetics, opioids, and other systemic medications. However, our tools to avoid central sensitization following nerve injury remain limited.

In recent years, an increased emphasis has been placed on the prevention and management of postinjury chronic pain states secondary to the military conflicts in the Middle East and around the globe. Between 2001 and 2010, more than 1600 US military personnel underwent amputation following military trauma.⁵ In addition, natural disasters, such as the 2010 Haitian earthquake, have created more than

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6000 amputees.⁶ Amputation surgery for medical and vascular disease also remains common, with a national rate of approximately 188 lower extremity amputations per 100,000 people.⁷ Given the combination of soft tissue, bone, and neurologic injury that occurs in the course of an amputation, initial management is often problematic; patients experience not only nociceptive pain but also acute neuralgia and occasionally the immediate onset of phantom limb pain.⁸

Similarly, thoracotomy is characterized by a high incidence of both severe acute pain and intractable postoperative pain.⁹ Poor analgesia following thoracotomy leads to poor chest wall mechanics, impaired cough, and subsequent respiratory and infectious complications. Given the preexisting tenuous pulmonary function of many thoracotomy patients, further decreases in pulmonary function may lead to significant additional morbidity.^{10,11}

An ideal perioperative analgesic regimen for surgeries, such as amputation and thoracotomy, would not only facilitate the immediate relief of suffering but would also reduce the burden of chronic postsurgical pain. Indeed, these goals seem physiologically linked given the correlation between the severity of perioperative pain and the prevalence of chronic pain.^{12–14} Despite these observational associations, the prevention of chronic postsurgical pain has been more difficult to accomplish than initially proposed.^{15–17} In this review, the authors discuss perioperative pain management techniques and modifiable risk factors to prevent chronic pain following amputation and thoracotomy.

CHRONIC POSTSURGICAL PAIN: AMPUTATION

Patients undergoing amputation experience a high level of both phantom and residual limb pain following surgery. Of these 2 complications, phantom limb pain has been more frequently discussed in the literature, with an estimated prevalence of 51% to 85%.^{18–21} Residual limb pain is also reported after amputation, with a frequency of 45% to 74%.^{22–24} Although residual limb pain phenomena, such as causalgia^{25,26} and neuroma,^{22,27} have been reported, they have not been systematically studied as separate entities in the residual limb.^{23,27–30} Nonetheless, distinction between the residual limb pain subtypes of neuroma, complex regional pain syndrome, and somatic pain is important for research and clinical care because all postamputation pain subtypes may not equally respond to a given therapy.

The appropriate treatment and prevention of postamputation pain is also of functional significance for patients. In a study of 2694 patients with amputations, 51% had phantom limb pain severe enough to impair lifestyle more than 6 days per month and 27% experienced pain more than 15 hours per day.^{20,31} The effects of residual limb pain may have even greater functional implications for the patients with amputations because of its impact on prosthetic use, ambulation, and rehabilitation.^{23,32}

In 1984, it was reported that fewer than 10% of patients with phantom limb pain obtained prolonged pain relief from medical treatments,³¹ and only limited progress has been made since that time.^{22,33} Surgical techniques, including dorsal root entry zone lesions, surgical sympathectomies, and spinal cord stimulation, have also been used.^{34–36} Currently, however, there is a lack of evidence to support the efficacy of these techniques.³⁷ There are promising data regarding improvements in phantom limb pain from body reimaging techniques with mirror box therapy; unfortunately, this intervention does not improve residual limb pain.³⁸

CHRONIC POSTSURGICAL PAIN: THORACOTOMY

Persistent post-thoracotomy pain is described as “pain along the incision site that persists or recurs after thoracotomy for at least two months following the surgical

procedure.”⁴ The cause of chronic pain following thoracotomy is undoubtedly similar to that following amputation. Neurologic injury at the time of surgery is likely the source of neuropathic pain, central sensitization, and persistent postsurgical pain in these patients.⁴

Up to 60% of thoracotomy patients report intractable pain a month after surgery and 30% to 50% report pain at 1 to 2 years.^{10,39,40} Many of these patients describe significant physical limitations and sleep disturbances months and even years after surgery.⁴¹ Similar to amputation pain, there is a strong correlation between severe perioperative pain and the incidence of chronic post-thoracotomy pain.^{42–46}

RISK FACTORS FOR DEVELOPING CHRONIC POSTSURGICAL PAIN

Although all patients who undergo amputation and thoracotomy experience peripheral nerve injury, not all develop persistent neuropathic pain. Therefore, predisposing risk factors must also be present for chronic postsurgical pain to develop. Regarding amputation, identified chronic pain risk factors include severe perioperative pain, psychosocial comorbidity, and genetic predisposition. In particular, the association between severe preoperative pain^{12,14,47–49} and postoperative pain^{13,46,50} and the development of chronic pain supports the critical importance of acute symptom management. Indeed, both pharmacologic evidence⁵¹ and radiologic demonstration^{52–54} suggests central nervous system reorganization and sensitization in patients with amputations. Logically, if the preoperative stimulus is removed, thereby reducing the pain memory, the risk of persistent pain following amputation may decrease. A similar correlation between severe perioperative pain and chronic pain is also well documented in patients undergoing thoracotomy.^{42–46} These observed associations between acute symptoms and chronic pain were part of the theoretical foundation behind the preemptive use of regional anesthesia before amputation and thoracotomy.^{15,48,55}

Psychosocial factors also have an impact on the risk of chronic postoperative pain. Comorbidities, such as preoperative anxiety^{56,57} and depression,^{22,47,58,59} correlate strongly with persistent postsurgical pain. A comprehensive preoperative evaluation to identify these risk factors may have an impact on reducing the burden of chronic postsurgical pain.⁶⁰

Gender and genetic risk factors are also increasingly appreciated as important to the development of chronic pain following surgery.⁶¹ Several gene single nucleotide polymorphisms that may contribute to the development of neuropathic pain have been identified. Detailed discussions of these genetic factors may be found in previous publications^{62,63} but are outside the scope of this review.

Given our current ability to identify predisposing factors for developing chronic postsurgical pain, we can now risk stratify patients who need more intensive multimodal therapy.⁶⁴ In subsequent sections, the authors focus on analgesic interventions that have been studied to reduce the incidence of persistent postsurgical pain.

ACUTE PAIN MANAGEMENT TECHNIQUES

Although there are evidence-based guidelines for acute pain management following thoracotomy,⁶⁵ there are no established guidelines for symptom management following amputation because of the inconsistent outcomes and methodological limitations of studies to date.⁶⁶ Surgical techniques, such as traction neurectomy and nerve implantation into muscle, may lessen the incidence of symptomatic neuromas.⁶⁷ However, these changes in technique have not significantly decreased the prevalence of chronic postamputation pain.²² Likewise, minimally invasive

thoracic surgery has not dramatically improved the incidence of moderate to severe pain following thoracotomy.⁶⁸

Many of the techniques studied in recent years for managing postamputation and post-thoracotomy pain have been initiated preoperatively.⁶⁹ This preemptive effect is designed to reduce nociceptive traffic to the spinal cord and central nervous system. In animal models, painful neuropathy can be attenuated with local anesthetic pretreatment^{70,71} or by aggressive early treatment of pain.¹⁴ Preemptive and perioperative therapies have been studied in an effort to reduce the burden of both acute and chronic postsurgical pain.

EPIDURAL ANALGESIA: AMPUTATION

Epidural analgesia is a common modality used to control acute pain at the time of amputation. Given the association between severe preoperative pain and chronic pain, investigators have hypothesized that aggressive perioperative pain control with epidural catheter infusion will also lessen the incidence of chronic postamputation pain. In a 1988 unblinded study of preemptive epidural analgesia, 25 patients in the epidural group reported dramatically reduced phantom limb pain at both 6 and 12 months when compared with controls.¹⁵ Similarly, in a 1994 case-controlled study, Jahangiri and colleagues⁷² observed only an 8% incidence of phantom limb pain in 24 patients treated with epidural bupivacaine, clonidine, and diamorphine compared with a 73% incidence in the control group treated with systemic opioids.

Unfortunately, these early successes were not repeated in later studies subjected to greater methodological rigor. In a 1997 prospective study, Nikolajsen and colleagues¹⁷ randomized patients to receive preoperative and postoperative epidural blockade or standard postoperative epidural analgesia. At 12 months, both groups had a significant incidence of phantom limb pain: 75% in the preoperative and postoperative block group and 69% in the standard epidural group. Although a nonepidural treatment group was not included in this study, the incidence of phantom limb pain in these 2 study arms was similar to the background prevalence of phantom limb pain noted in other studies.^{21,24,73} In a follow-up article, Nikolajsen and colleagues⁷⁴ examined the effect of preoperative and intraoperative epidural analgesia on stump sensitization after amputation. Again, they found no significant improvements. These findings are consistent with other clinical studies demonstrating that the timing of an analgesic intervention is not of critical importance.⁶⁹

The current de-emphasis of the preemptive analgesia paradigm, however, has not lessened the significance placed on effective pain relief at the time of surgery. Indeed, the importance of successful analgesia is further supported by the 2011 publication by Karanikolas and colleagues⁷⁵ assessing epidural versus systemic analgesia in 65 patients undergoing amputation. Nearly all patients receiving epidural infusion or effective systemic analgesia saw a reduction in the prevalence of phantom limb pain at 6 months when compared with the controls treated with nurse-delivered intramuscular opioids. This article supports the concept that the success of analgesia may be more important than the specific technique used.

EPIDURAL ANALGESIA: THORACOTOMY

Similar to the interventions used for amputation surgery, epidural infusion has also been the gold standard for pain relief following thoracic surgery.⁷⁶ Thoracic epidural analgesia provides superior postoperative pain control when compared with parenteral opioids^{77,78} and also facilitates early extubation, rehabilitation, and decreased perioperative complications.⁷⁹ The Procedure Specific Postoperative

Pain Management working group (www.postoppain.org) recommends thoracic epidural or paravertebral blocks for thoracic surgery as the first-line approach.

Despite the documented efficacy of thoracic epidural analgesia in the perioperative setting, the technique still fails in a significant number of patients.⁸⁰ The reason for this is unclear, and multiple hypotheses include catheter malposition, opioid tolerance, or poor drug spread to nerves located on the operative side.^{81–84} Currently, there is limited evidence to support the notion that epidural analgesia reduces the incidence of chronic post-thoracotomy pain.

REGIONAL ANALGESIA: AMPUTATION

As an alternative to epidural analgesia, several trials of perineural catheters have been conducted in an effort to improve both acute and chronic pain symptoms following amputation. Initial studies of surgically placed perineural catheters were encouraging. In 1991, Malawer and colleagues⁸⁵ reported excellent perioperative analgesia with nerve sheath catheters in patients with amputations, and Fisher and Meller¹⁶ described the complete absence of phantom limb pain in 11 patients treated with this technique.

Additional trials of this technique, however, did not reproduce these initial positive results. In 1994, Elizaga and colleagues⁸⁶ observed no significant improvement in acute or chronic pain in patients treated with surgically placed catheters. Other studies have reported either modest⁸⁷ or no improvement in the incidence of phantom limb pain.⁸⁸ It is also notable that surgically placed perineural catheters seem to provide inferior acute analgesia when compared with other regional anesthesia and epidural techniques.⁸⁹ The inadequate perioperative analgesia may be secondary to the distal placement of the catheter with minimal blunting of sensation at the surgical site. It is unknown whether the reduction in acute analgesia from surgical catheters has implications for longer-term postsurgical pain.

Although the previously mentioned studies of surgically placed perineural catheters provided equivocal results for managing postamputation pain, other percutaneous catheter insertion techniques are now commonly used by anesthesiologists and provide some potential advantages.⁹⁰ First of all, catheters may be placed preoperatively and used in a preemptive fashion. Secondly, and more importantly, the catheters may be placed in a location proximal to the incision, improving postoperative analgesia.

Previous studies gave sporadic reports of effective management of amputation pain using proximal perineural catheters.^{91–93} More recently, Borghi and colleagues⁹⁴ evaluated this technique in a more systematic manner and found that prolonged perineural catheter use provided effective acute analgesia and long-term reduction of phantom limb pain. Notable aspects of this study were the lack of preoperative infusion and the prolonged duration of postoperative catheter use (median catheter duration of 30 days). Although not a randomized trial, the investigators did find only a 16% incidence of phantom limb pain at 12 months follow-up. These results have not yet been duplicated but are quite encouraging.

REGIONAL ANALGESIA: THORACOTOMY

Similar to perineural catheter infusions for amputation pain, paravertebral nerve blockade also involves the delivery of local anesthetic to nerves after they exit the spinal canal. Single-injection techniques at multiple dermatomes and continuous paravertebral catheters are generally used to manage pain from thoracotomy surgery. The classic method uses a loss-of-resistance technique; however, nerve stimulator

localization⁹⁵ and ultrasound techniques are also well described.^{96–98} Ultrasound guidance improves accuracy of paravertebral catheter placement and minimizes the risk of pleural puncture.^{99,100} Karmarkar and Richardson^{101,102} provide additional details about these techniques.

Recent studies suggest that paravertebral nerve block provides comparable analgesia to epidural infusion with greater hemodynamic stability¹⁰³ and a better short-term side-effect profile.¹⁰⁴ The side effects associated with thoracic paravertebral blockade are generally low, although local anesthetic toxicity, block failure, bleeding, and pleural puncture may occur.^{101,105,106} It is thought that pulmonary function is preserved with paravertebral block, subsequently decreasing pulmonary morbidity.^{3,107–109} Thus, paravertebral blockade along with epidural infusion is still recommended.

SYSTEMIC MULTIMODAL ANALGESIA

Despite the recent emphasis placed on the perioperative use of epidural analgesia and peripheral nerve blockade, these techniques alone may not be sufficient for the prevention of chronic postsurgical pain. Circulating humoral inflammatory factors also induce central sensitization and neuropathic pain,^{110,111} providing scientific justification for using multimodal systemic analgesia. Multimodal strategies use concurrent therapies in an effort to maximize pain relief and minimize side effects, particularly those related to opioid analgesics.¹¹² Although opioid analgesics remain an important part of the acute pain protocol for amputation and thoracic surgery, their singular use is often not sufficient to provide effective systemic analgesia. In this review, the authors discuss adjuvant analgesics and novel nonopioid pain control strategies.

Nonsteroidal Antiinflammatory Drugs

Nonsteroidal antiinflammatory drugs (NSAIDs) have been extensively investigated in the perioperative period, and their use improves analgesia, reduces opioid requirements, and reduces opioid-related side effects.¹¹³ Additionally, the question of preemptive analgesia from preoperative NSAID administration has been investigated in more than 20 trials. However, preoperative dosing improved symptom management in only 2 of these trials when compared with intraoperative and postoperative dosing, indicating that there is likely little or no preemptive effect from these drugs.⁶⁹

Cyclooxygenase-2 (COX-2) inhibitors are sometimes preferred in the perioperative period given their decreased effect on platelet function. Similar to other NSAIDs, the COX-2 inhibitor celecoxib demonstrates improvement in acute analgesia with an opioid-sparing effect but no significant preemptive analgesic effect.^{114–116} Celecoxib has demonstrated efficacy as part of a multimodal strategy for thoracic surgery.¹¹⁷ Studies related to NSAID efficacy following amputation are lacking, but these analgesics should be considered given their documented effectiveness for acute pain. However, there is no current evidence that NSAID use prevents either chronic postamputation or post-thoracotomy pain.

Acetaminophen

Oral acetaminophen has enjoyed long-term use for managing acute pain, and intravenous (IV) acetaminophen has recently been approved in the United States. Although both forms have been used in the perioperative period, the IV formulation may have some advantages given its reliable pharmacokinetics and ease of administration.^{118,119}

Because acetaminophen improves acute analgesia in patients undergoing thoracotomy, it is increasingly being used in the perioperative period, except in patients with significant liver disease.¹²⁰ Although there are concerns about the safety of chronic acetaminophen use, acute administration of up to 4 g/d seems to be safe in most patients.¹²¹ Similar to NSAIDs, however, no studies have demonstrated that acetaminophen reduces chronic postamputation or post-thoracotomy pain. Nonetheless, given its minimal effect on platelet aggregation, perioperative bleeding, and renal function,¹²² acetaminophen should be strongly considered in the perioperative setting.

Gabapentinoids: Gabapentin/Pregabalin

There has been significant interest in the use of gabapentinoids for neuropathic pain since their 1993 release in the United States. Because these drugs can inhibit Ca^{2+} currents and reduce neurotransmitter release associated with neural sensitization,¹²³ they have demonstrated efficacy in multiple neuropathic pain conditions.^{124,125}

Gabapentin and pregabalin have been studied as a preemptive measure before surgery with evidence of decreased acute pain, opioid consumption, and improvement in opioid-related side effects.^{126–128} Additionally, gabapentin is effective in reducing the severity of chronic phantom limb pain.³³ Despite the demonstrated efficacy of gabapentinoids in acute and chronic neuropathic pain, they have not been shown to prevent chronic phantom limb pain when given in the immediate postoperative period.¹²⁹ Although their use following amputation may be appropriate given their beneficial effect on acute postoperative pain, future research is needed to establish optimal timing, dosing, and efficacy of perioperative gabapentinoids.^{128,130,131}

Clonidine

Clonidine, an α_2 adrenergic agonist, plays a potential role in the treatment of neuropathic pain because of the expression of $\alpha_2\text{A}$ receptors at the site of nerve injury¹³² as well as on local infiltrating macrophages and lymphocytes.¹³³ Clonidine administration decreases the local expression of inflammatory cytokines, such as $\text{TNF-}\alpha$ and $\text{IL-1}\beta$, and improves hypersensitivity following nerve injury.¹³⁴ Epidural and perineural clonidine have also been studied as a therapy for neuropathic pain¹³⁴ and have been used clinically in the treatment of chronic postamputation pain.^{135,136} Because $\alpha_2\text{A}$ -adrenoceptors and inflammatory cytokines play important roles in the production of postamputation chronic pain, clonidine deserves further investigation. It is generally well tolerated, but its clinical use is occasionally limited by dose-dependent side effects, such as hypotension and sedation.¹³⁷

Ketamine

Ketamine is an antagonist of the N-methyl D-aspartate receptor known to be involved in central sensitization and neuropathic pain.¹³⁸ It has been used in the treatment and prevention of chronic pain following nerve injury, although randomized controlled efficacy trials are still lacking.¹³⁹ Ketamine has been investigated as a systemic drug^{51,140} and an epidural drug¹⁴¹ for amputation surgery and it has been shown to reduce stump sensitivity in the immediate postoperative period.¹⁴¹ Although ketamine has also been found to reduce acute hyperalgesia and allodynia when given at the time of thoracic surgery,¹⁴² it is not effective for treating chronic postamputation pain¹⁴¹ or post-thoracotomy pain.¹⁴³

SUMMARY AND FUTURE DIRECTIONS

Growing evidence suggests that multimodal analgesia, using a combination of catheter-based techniques^{94,144} and systemic analgesics,^{112,145,146} reduces the risk of chronic postsurgical pain. Comprehensive therapy is particularly important for patients undergoing high-risk surgeries, such as amputation and thoracotomy. With the recent demonstration that effective acute pain management, regardless of the method used, decreases the prevalence of phantom limb pain at 6 months,⁷⁵ we now have the scientific justification and the ethical obligation to treat these patients with the multiple tools at our disposal. Furthermore, because prolonged perineural catheter infusions may reduce the burden of postamputation pain,⁹⁴ we must reevaluate the postoperative treatment period. Therefore, rather than several days of recovery, we may need to consider prolonged therapies during the time of neurologic plasticity. If we can alter this postoperative remodeling process, we will have an additional tool to reduce the incidence of chronic postsurgical pain.

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ASA Abstract 1

Title: Pre-operative dexamethasone decreases the development of chronic mechanical allodynia in a mouse tibial spared nerve injury model.

AUTHOR(S):

T. Van de ven¹, H. Hsia¹, T. Buchheit¹, H. Sheng¹, A. D. Shaw¹

1. Duke University, Durham, NC

Background:

Patients undergoing certain surgical procedures, such as thoracotomy or amputation, are at high risk for the development of chronic neuropathic pain. A large percentage of patients undergoing these major surgical procedures continue to have pain at the surgical site one year following the procedure and current therapy is limited.

New research suggests that pro-inflammatory responses to nerve injury play an important role in the development of chronic neuropathic pain. After peripheral nerve injury, macrophages, neutrophils, lymphocytes and mast cells infiltrate the injured nerve and release inflammatory mediators which cause further damage and can sensitize nociceptive receptors leading to peripheral and central sensitization.³ Given the immunomodulatory and anti-inflammatory properties of dexamethasone, it deserves further research as a candidate drug for prevention of the development of chronic neuropathic pain. For this study, a spared nerve injury (SNI) mouse model was used to test dexamethasone as a therapeutic and preventative intervention in the development of chronic mechanical allodynia.^{1,2}

Methods:

After IACUC approval, 30 C57/Bl6 mice were divided into three groups. 5 mice were used as the nerve injury control group and received a spared tibial nerve injury without pharmacological intervention. 5 mice were used as sham controls in which dissection down to the sciatic nerve and subsequent branches was accomplished, but no ligation and transection of nerves was performed. 5 mice were used as the experimental intervention group where intraperitoneal dexamethasone (10mg/kg) was administered 1 hour prior to surgical ligation and transection of the sural and common peroneal nerve with sparing of the tibial nerve. All surgery was performed under isoflurane anesthesia.

All mechanical threshold testing, including baseline and all subsequent post-surgical measurements, was performed with an electronic von Frey anesthesiometer (Life Science 2390 series). Baseline mechanical threshold testing was performed prior to surgery. Post-surgical measurements were performed starting on post-operative day 3 and every following third day, finishing on post-operative day 21.

Results:

Out of the three groups, only the SNI group demonstrated a statistically significant decrease in mechanical paw withdrawal thresholds on the operative side and maximal effect was observed on day 15. For the SNI group; ipsilateral compared to contralateral paw withdrawal thresholds: 1.21g +/-

0.127g vs. 5.32g \pm 1.21g, $P = 0.041$, respectively. For the sham control group; ipsilateral compared to contralateral paw withdrawal thresholds: 5.15g \pm 0.25g vs. 4.82g \pm 0.34g, $P = 0.251$, respectively. For the dexamethasone experimental group; ipsilateral compared to contralateral paw withdrawal thresholds: 5.392 \pm 0.299 vs. 5.496g \pm 0.76g, $P = 0.784$.

Conclusion:

Given these results, dexamethasone likely prevents the development of chronic mechanical allodynia by suppressing inflammatory processes leading to peripheral and central sensitization.

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1. Shields S, Eckert W, Basbaum A. *Spared Nerve Injury Model of Neuropathic Pain in the Mouse: A Behavioral and Anatomic Analysis*. The Journal of Pain, Vol. 4, No 8: pp 465-470
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3. Bastos L, Medeiros D, Vieira R, Watkins L, Coelho M, Moraes M. *Intraneural dexamethasone applied simultaneously to rat sciatic nerve constriction delays the development of hyperalgesia and allodynia*. Neuroscience Letter 510 (2012) 20-23

ASA Abstract 2

Title: Sub-anesthetic ketamine prior to nerve lesion reduces the development of chronic neuropathic pain in a mouse tibial spared nerve injury model.

AUTHOR(S):

T. Van de ven¹, H. Hsia¹, T. Buchheit¹, H. Sheng¹, A. D. Shaw¹

1. Duke University, Durham, NC

Background:

A common complication of nerve injury is the development of neuropathic pain. Patients undergoing surgical procedures, especially those requiring a large incision or amputation, are at high risk for the development of chronic neuropathic pain. A large percentage of patients undergoing these major surgical procedures continue to have pain at the surgical site one year following the procedure and current therapy is limited. The purpose of this study is to utilize a spared nerve injury (SNI) mouse model to test ketamine as a therapeutic and preventative intervention in the development of chronic neuropathic pain. This spared nerve model for neuropathic pain has been previously validated by prior research.¹

NMDA receptor activity is thought to play a major role in central sensitization involved in the development of chronic neuropathic pain.² Ketamine, given as an anesthetic dose in a mouse SNI model, prevents the development of changes in mechanical thresholds.¹ This study postulated that a sub-anesthetic dose prior to SNI would also prevent the development of changes in mechanical threshold associated with a neuropathic pain phenotype.

Methods:

After IACUC approval, 25 C57/Bl6 mice were divided into three groups. 5 mice were used as the nerve injury control group and received a spared tibial nerve injury without pharmacological intervention. 5 mice were used as sham controls in which dissection down to the sciatic nerve and subsequent branches was accomplished, but no ligation and transection of nerves was performed. 5 mice were used as the experimental intervention group where subcutaneous ketamine (20mg/kg) was administered in between the shoulder blades 1 hour prior to surgical ligation and transection of the sural and common peroneal nerve with sparing of the tibial nerve. All surgery was performed under isoflurane anesthesia.

All mechanical threshold testing, including baseline and all subsequent post-surgical measurements, was performed with an electronic von Frey anesthesiometer (Life Science 2390 series). Baseline mechanical threshold testing was performed prior to surgery. Post-surgical measurements were performed starting on post-operative day 3 and every following third day, finishing on post-operative day 21.

Results:

Out of the three groups, only the SNI group demonstrated a statistically significant decrease in mechanical paw withdrawal thresholds on the operative side and maximal effect was observed on post-

operative day 15 (POD 15). For the SNI group; ipsilateral compared to contralateral paw withdrawal thresholds: 1.21g +/- 0.127g vs. 5.32g +/- 1.21g, $P = 0.041$, respectively on POD 15. For the sham control group; ipsilateral compared to contralateral paw withdrawal thresholds: 5.15g +/- 0.25g vs. 4.82g +/- 0.34g, $P = 0.251$, respectively on POD 15. For the ketamine experimental group; ipsilateral compared to contralateral paw withdrawal thresholds: 4.512g +/- 0.637g vs. 5.36g +/- 0.955g, $P = 0.13$ on POD 15.

Conclusion:

Given these results, a sub-anesthetic dose of ketamine 20mg/kg prior to spared nerve injury likely attenuates or prevents the development of mechanical allodynia most likely through NMDA receptor antagonism, which inhibits central sensitization.

References:

1. Shields S, Eckert W, Basbaum A. *Spared Nerve Injury Model of Neuropathic Pain in the Mouse: A Behavioral and Anatomic Analysis*. The Journal of Pain, Vol. 4, No 8: pp 465-470
2. Zhou H, Chen S, Pan H. *Targeting N-methyl-D-aspartate receptors for treatment of neuropathic pain*. Expert Rev Clin Pharmacol. 2011 May 1; 4(3): pp 379-388

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Control/Tracking Number : 12-A-3680-ASAHQ

Activity : Abstract

Current Date/Time : 3/27/2012 10:03:30 AM

TITLE:

Pain candidate pathway prioritization using interspecies plasma metabolomics.

AUTHOR(S):

A. D. Shaw¹, H. Hsia¹, T. Van de ven¹, T. Buchheit¹, J. Lucas¹, M. McDuffie², C. Buckenmaier²;

¹Duke University, Durham, NC, ²WRNMMC, Bethesda, MD.

AFFIRMATIONS:

Affirmations (Complete):

- *: I agree to the above statements
- *: I agree to the above statements
- *: Accept
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SESSION CATEGORY:

3.1 CHRONIC AND CANCER PAIN - Basic Science

QUESTIONNAIRE:

Questionnaire (Complete):

Please select: No, do not consider my abstract for the 2012 ANESTHESIOLOGY Journal Symposium

Please select: The presenting author is NOT a resident or fellow.

Will you be able to participate in the Resident Research Forum to be held on Saturday, October 15th at 1:00PM? Not applicable

Funding Information: CDMRP award #DM102142 to Dr Shaw

ABSTRACT:

Background: Persistent pain after surgical nerve damage is a significant problem, affecting patients undergoing many different procedures. The biological pathways responsible are poorly characterized, and little progress has been made in the field of novel analgesic development. In order to prioritize the biological pathways of relevance we have compared the plasma metabolomes of humans with persistent pain after surgical amputation and C57/Bl6 mice undergoing spared nerve injury. We hypothesize that pathways that are demonstrably important in *both* species represent high priority candidates both for further mechanistic study, and also for therapeutic target discovery.

Methods: After IRB and IACUC approval we are studying 20 human subjects with persistent neuropathic pain who had sustained a traumatic amputation in the prior 3-18 months, and 20 C57/Bl6 mice who underwent sciatic spared nerve injury in the prior 3 weeks.

Human chronic pain phenotypes were adjudicated by committee, mice chronic pain phenotypes were measured using electronic Von Frey

apparatus and plasma samples were drawn for metabolomic analysis from both humans and mice. Assays are conducted by Metabolon Inc, Raleigh, NC. Data are compared in order to identify pathways of relevance that are either convergent across both species, or show significant divergence between humans and mice. In general, metabolite fold increase or decrease is compared between human and mouse phenotypes using 2-way ANOVA, and multiple comparisons controlled using false discovery. Dimensionality reduction is achieved using principal components analysis.

Results: Metabolomic analysis identifies over 300 different metabolites, and many more unknown compounds. Some biochemical pathways are convergent between humans and mice, whereas others are restricted to a single species. Data will be shown using a heatmap (fold change) diagram, and annotated Venn diagrams of overlapping pathway significance.

Conclusions: We are conducting comparative biological investigations of persistent pain phenotypes in two evolutionarily distant species in order to detect pathways of biological relevance following peripheral nerve injury. We will use these data to inform further proteomic and genomic experiments probing ever deeper into the preserved, but maladapted, inflammatory response to nerve injury.

SUMMARY:

We present a comparative biological study of the human and murine plasma metabolomic response to peripheral nerve injury.

Status: Complete

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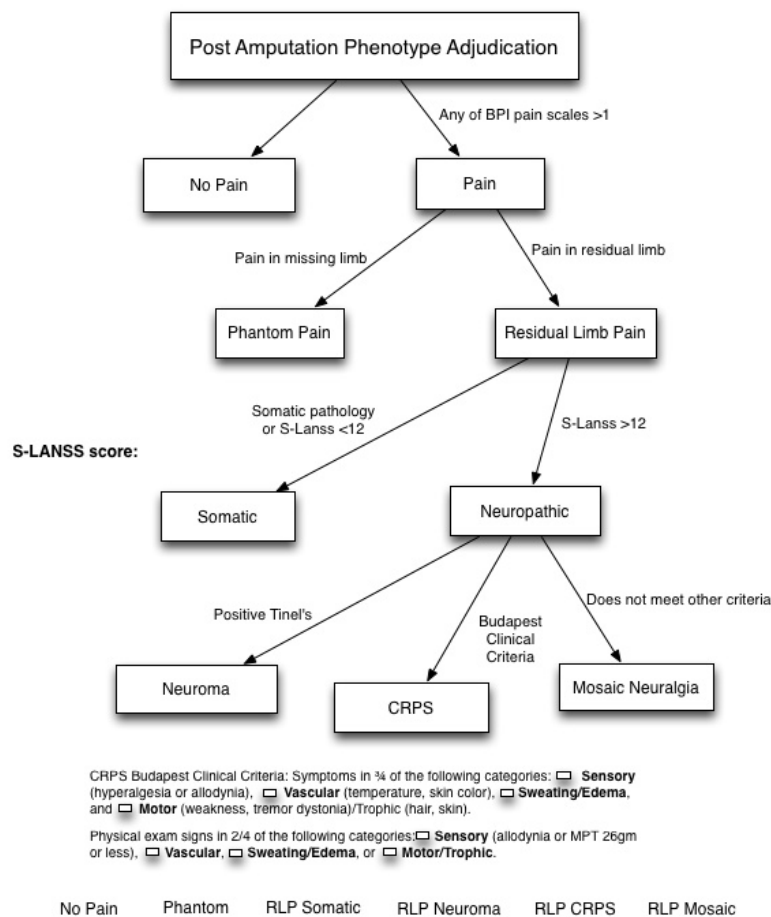
Veterans Integrated Pain Evaluation Research (VIPER): Post-amputation Pain Phenotypes in Injured Military Service Personnel

Thomas Buchheit MD, Thomas VandeVen MD PhD, Mary McDuffie RN, Hung-Lun John Hsia MD,
COL Chester “Trip” Buckenmaier MD, and Andrew Shaw MB FRCA

Background

Post-amputation pain is present in more than 50% of injured military service members after amputation.¹ Although distinct pain syndromes such as neuroma and complex regional pain syndrome have been described^{2,3}, most studies discriminate only between phantom and residual limb pain.⁴ Similar to advances that have been made with other chronic diseases after diagnostic improvements,⁵ classifying pain phenotypes may ultimately lead to more disease-specific and effective therapies. With this goal, we are performing a collaborative study (Veterans Integrated Pain Evaluation Research (VIPER)) between Duke University and Walter Reed National Military Medical Center (WRNMMC) of injured military service personnel who have undergone previous traumatic amputation. We report the assessment and phenotypic adjudication of the first 15 patients enrolled in VIPER, who represent the initial pilot cohort.

METHODS



After IRB approval, the VIPER pilot clinical cohort was assessed using several well established and validated questionnaire instruments including the Brief Pain Inventory (BPI), Self-Reported Leeds Assessment of Neuropathic

Symptoms and Signs Pain Scale (S-LANSS), Complex Regional Pain Syndrome questions (Budapest Clinical Criteria) phantom and residual limb pain questionnaires.

These questionnaire instruments were applied to each case as part of a formal endpoint adjudication process as required by the VIPER protocol in order to discriminate between distinct pain phenotypes. Using an algorithm previously reported by our group ⁴, phantom pain was first distinguished, and subsequently, residual limb pain was sub-categorized into 1) Somatic 3) Neuroma/Neuritis 4) CRPS or 5) Mosaic Neuralgia (neuropathic pain not otherwise specified).

RESULTS

Using these validated assessment tools, we were able to successfully discriminate between multiple categories of post-amputation pain in this preliminary cohort. We found that 86% described phantom pain, 13% noted residual limb somatic pain, 33% residual limb neuroma pain, 7% residual limb CRPS pain, and 20% described neuralgic limb pain not otherwise specified (Mosaic neuralgia). Importantly, there was significant overlap between phantom limb pain and residual limb neuralgic pain.

Patient	Chronic Pain	Phantom Pain	Residual Limb Pain			
			RLP Somatic	RLP Neuroma	RLP CRPS	RLP Mosaic
1	yes	yes		yes		
2	yes	yes	yes			
3	yes			yes		
4	yes	yes				yes
5	yes	yes		yes		
6	yes	yes				yes
7	no					
8	yes	yes			yes	
9	no					
10	yes	yes	yes			
11	no					
12	yes	yes		yes		
13	yes	yes				
14	yes	yes		yes		
15	yes	yes				yes

Summary

This preliminary research describes significant phenotypic complexity within the post-amputation pain syndromes, including several different subtypes of residual limb neuropathic pain. Further cohort analyses will allow for better diagnostic discrimination between post-amputation pain subtypes, and may facilitate targeted future therapies.

1. Reiber GE, McFarland LV, Hubbard S, et al. Servicemembers and veterans with major traumatic limb loss from Vietnam war and OIF/OEF conflicts: survey methods, participants, and summary findings. *J Rehabil Res Dev.* 2010;47(4):275-297.
2. Sehirlioglu A, Ozturk C, Yazicioglu K, Tugcu I, Yilmaz B, Goktepe AS. Painful neuroma requiring surgical excision after lower limb amputation caused by landmine explosions. *International orthopaedics.* Apr 2009;33(2):533-536.
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4. Lindsay DR, Pyati S, Buchheit TE, Shaw A. Residual limb pain: more than a single entity? *Anesthesiology.* Jan 2012;116(1):224.
5. Jaglowski S, Jones JA. Choosing first-line therapy for chronic lymphocytic leukemia. *Expert Rev Anticancer Ther.* Sep 2011;11(9):1379-1390.

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Control/Tracking Number : 12-A-4415-ASAHQ

Activity : Abstract

Current Date/Time : 3/31/2012 9:06:33 PM

TITLE:

Veterans Integrated Pain Evaluation Research (VIPER) Pilot Cohort: Feasibility of Studying Combat Amputation Pain

AUTHOR(S):

A. D. Shaw¹, T. Buchheit¹, T. Van de ven¹, H. Hsia¹, M. McDuffie², C. Buckenmaier²;

¹Duke University, Durham, NC, ²WRNMMC, Bethesda, MD.

AFFIRMATIONS:

Affirmations (Complete):

- *: I agree to the above statements
- *: I agree to the above statements
- *: Accept
- *: Yes
- *: No animal subjects were involved in the research
- *: Yes, I have IRB or IACUC approval
- *: I agree to the above statements

SESSION CATEGORY:

3.2 CHRONIC AND CANCER PAIN - Clinical

QUESTIONNAIRE:

Questionnaire (Complete):

Please select: No, do not consider my abstract for the 2012 ANESTHESIOLOGY Journal Symposium

Please select: The presenting author is NOT a resident or fellow.

Will you be able to participate in the Resident Research Forum to be held on Saturday, October 15th at 1:00PM? Not applicable

Funding Information: CDMRP award #DM102142 to Dr Shaw

ABSTRACT:

Veterans Integrated Pain Evaluation Research (VIPER) Pilot Cohort: Feasibility of Studying Combat Amputation Pain

Andrew Shaw MB FRCA, Thomas Buchheit MD, Thomas VandeVen MD PhD, Mary McDuffie RN, Hung-Lun John Hsia MD, Chester Buckenmaier MD

BACKGROUND

We are currently enrolling military service personnel returning from Operation Iraqi Freedom (OIF), Operation Enduring Freedom (OEF) and Operation New Dawn (OND) in a study of the determinants of persistent pain after devastating limb injury in the absence of traumatic brain injury. To date (March 2012) we have enrolled 20 soldiers (the VIPER pilot cohort), who underwent devastating peripheral limb injury

between 3 and 18 months previously, and here we report data regarding the characteristics of their pain syndromes. This cohort represents 13% of the total planned enrollment of 150 patients.

METHODS

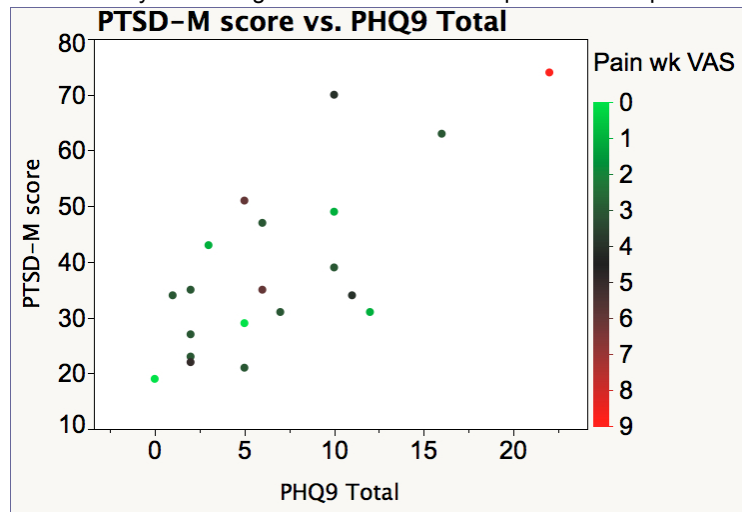
After IRB approval, the VIPER pilot clinical cohort was assessed using several well established and validated questionnaire instruments including the Brief Pain Inventory (BPI), Self-Reported Leeds Assessment of Neuropathic Symptoms and Signs Pain Scale (S-LANSS), Post Traumatic Stress Disorder (Military) scale, phantom and residual limb pain questionnaires. Additionally, soldiers were interviewed regarding their pain symptomatology, their pain perceptions, and measurements made of their mechanical detection and pain thresholds. Descriptive data are reported for all 20 patients; no comparison data are reported because of the small sample size and the fact that this pilot cohort primarily represents a study of the feasibility of enrolling these soldiers as they rehabilitate from devastating combat injury. Blood samples for biomarker and therapeutic discovery studies have also been collected, details of which are reported in a separate abstract.

RESULTS

18 of 20 (90%) patients suffered lower limb amputation and 2 of 20 (10%) upper limb amputation. 17 of 20 (85%) patients reported phantom pain, and 15 of 20 (75%) reported residual limb (stump) pain. 7 patients reported that these symptoms interfere significantly with their daily activities. Mean (SD) VAS score across the whole cohort was 3.2 (2.2), mean neuropathic score was 13.6 (7.7), mean PTSD score was 38.9 (15.9) and mean PHQ 9 score was 6.9 (5.6). There was no statistically significant effect of regional anesthetic catheter use, age, ethnicity, race, smoking status, type of injury, type of amputation or body mass index on either the incidence or severity of pain; however the p value for regional catheter use was 0.06 suggesting that as the sample size increases this variable may develop significance. The figure shows PTSD, PHQ 9 and VAS scores for all 20 patients.

Summary

In this pilot cohort of 20 patients who sustained devastating peripheral limb combat injuries, the incidence of chronic phantom and/or residual limb pain was greater than 75%. There is considerable overlap between phantom and residual limb pain, and both interfere significantly with patients' daily activities. The incidence of PTSD symptomatology is high in this military population; but whether or not this is important for the pain subtype is presently uncertain. The early use of regional catheters for the prevention of persistent pain in the



traumatic amputation setting may warrant further study.

SUMMARY:

We report the initial 20 patient pilot cohort of an ongoing study of the clinical and molecular determinants of persistent pain after combat amputation.

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Dexamethasone Attenuates Neuropathic Pain Behavior

TJ Van de Ven, HL Hsia, H Sheng, D Macleod, TE Buchheit, AD Shaw
Department of Anesthesiology, Duke University Medical Center/Durham VAMC, Durham, NC USA



Introduction

- Neuropathic pain is a common complication of nerve injury.
- Proposed mechanisms include both local and systemic inflammation.
- Dexamethasone is a known anti-inflammatory agent often used in the operating room.
- Plasma metabolomics are a useful cross-species pathway discovery tool.

Hypotheses

- Dexamethasone attenuates the development of neuropathic pain behavior
- Spared nerve injury and treatment with dexamethasone cause reproducible metabolic changes
- Differentially regulated metabolites can serve as biomarkers of pain susceptibility and can inform pathway discovery.

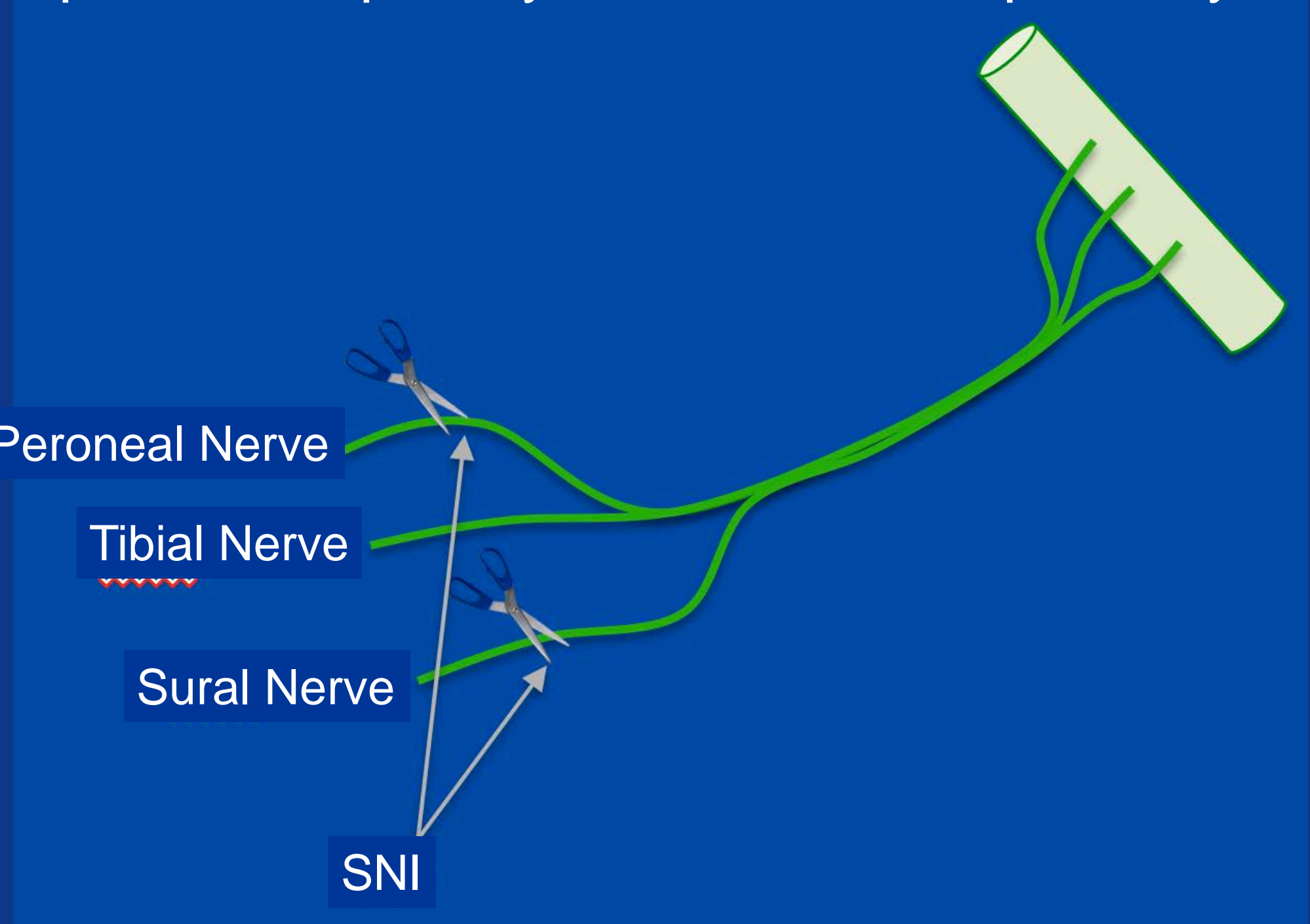
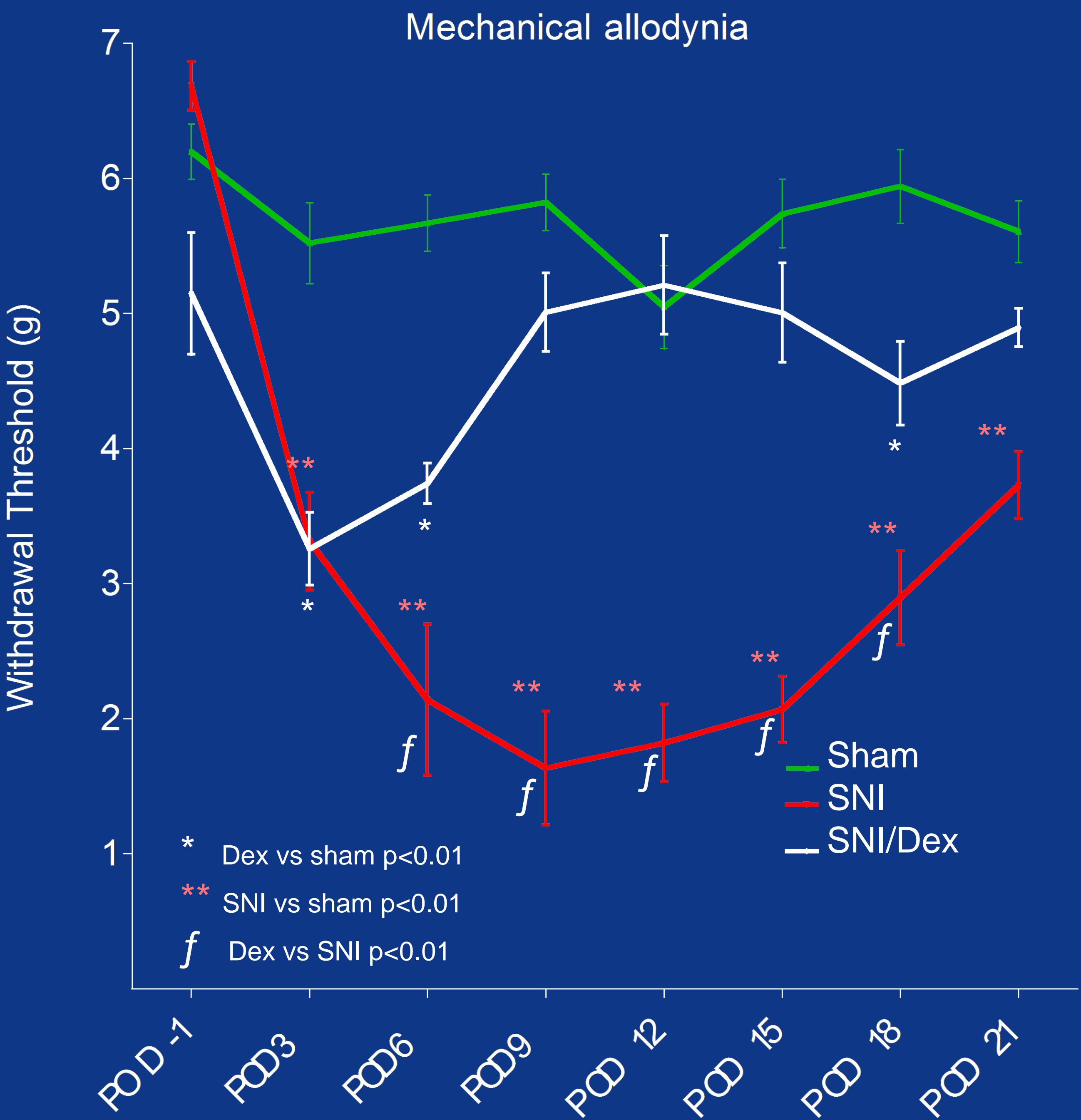


Figure 2: Spared Nerve Injury Model

Group	n	Description
Mouse CTRL	5	Sham
Mouse INT	15	SNI
Mouse TRT	5	SNI/Dex

Figure 3: Metabolomics Study Overview

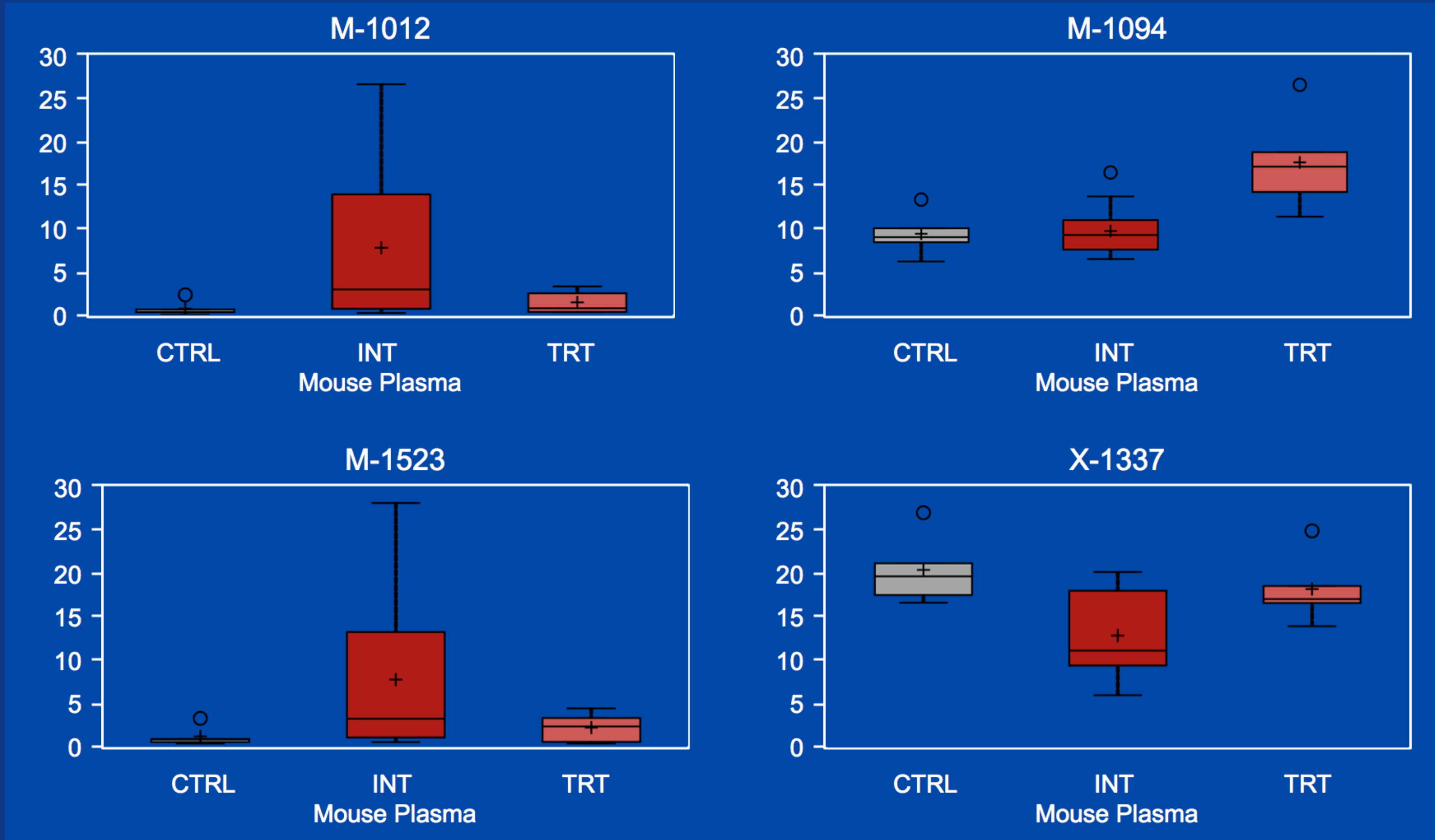


Figure 4: Metabolite concentration box plots

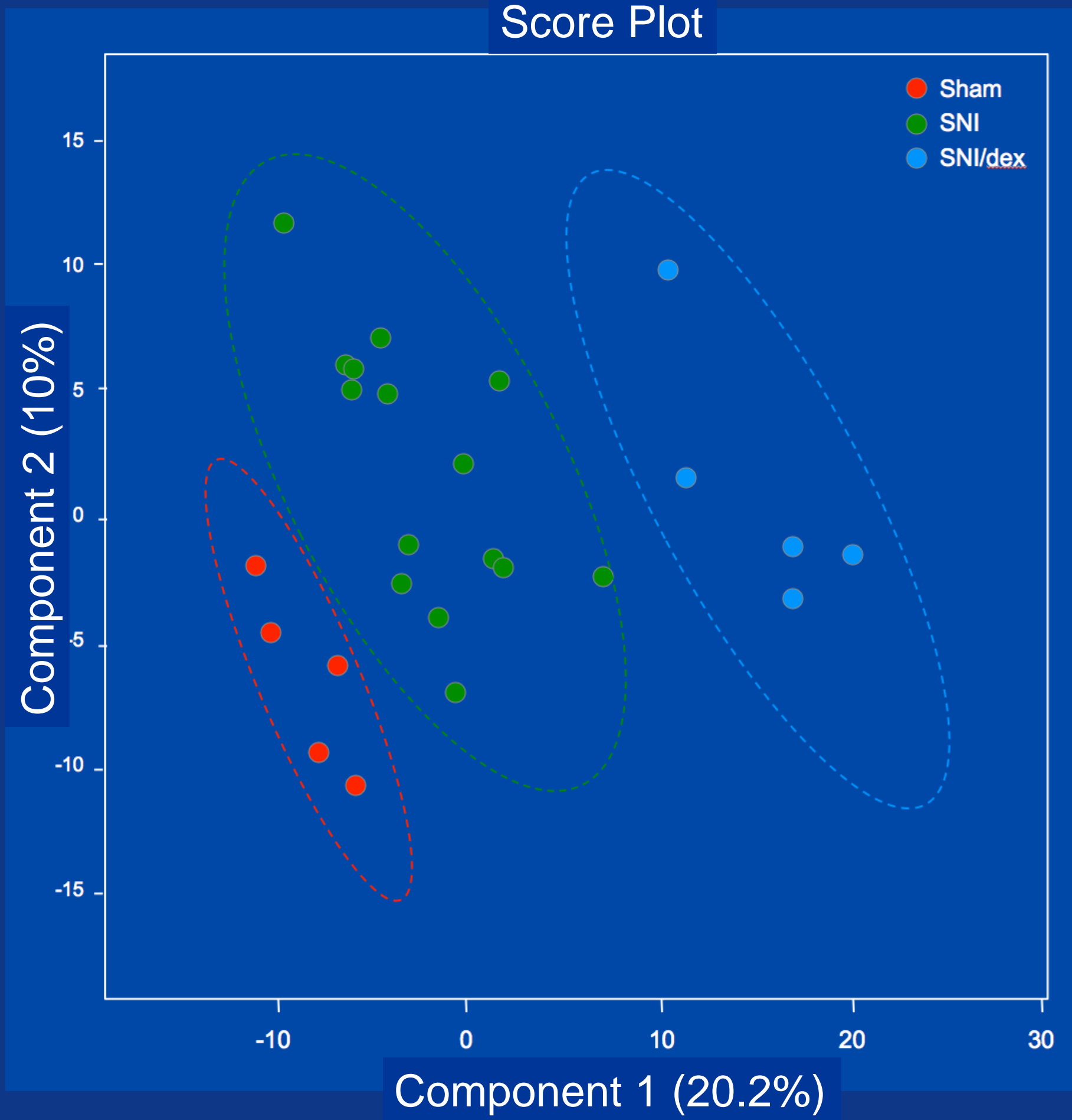


Figure 5: PLS-DA score plot showing class separation

Methods

- After IACUC approval, 30 C57/Bl6 mice were randomly allocated into three groups:
 - Spared nerve injury (SNI), N=10
 - Sham surgery, N=10
 - Dexamethasone/SNI, N=10
- Mechanical withdrawal threshold was measured using an electronic von Frey anesthesiometer.
- Blood plasma was obtained and unbiased metabolomics performed at Metabolon, Inc.

Results

- The SNI group showed a statistically significant ($P < 0.01$) decrease in paw withdrawal threshold (PWT) on the surgical hind paw compared with sham.
- Compared to the SNI group, the treatment group demonstrated statistically significant ($P < 0.01$) increases in PWT.
- 51 metabolites are differentially regulated in sham vs. SNI operated mice. ($p < 0.05$ FDR corrected)
- 177 metabolites are differentially regulated between SNI and SNI/Dexamethasone treated mice. ($p < 0.05$ FDR corrected)
- Multivariate analysis shows significant differential clustering of all three experimental groups

Conclusions

- Dexamethasone attenuates neuropathic pain behavior.
- SNI and dexamethasone treatment produce reproducible metabolic changes in mice.

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Supported by NIH T32 #2T32GM008600-16 and DMRDP Grant #DM102142



Ketamine Attenuates Neuropathic Pain Behavior

TJ Van de Ven, HL Hsia, H Sheng, D Macleod, TE Buchheit, AD Shaw

Department of Anesthesiology, Duke University Medical Center/Durham VAMC, Durham, NC USA



Introduction

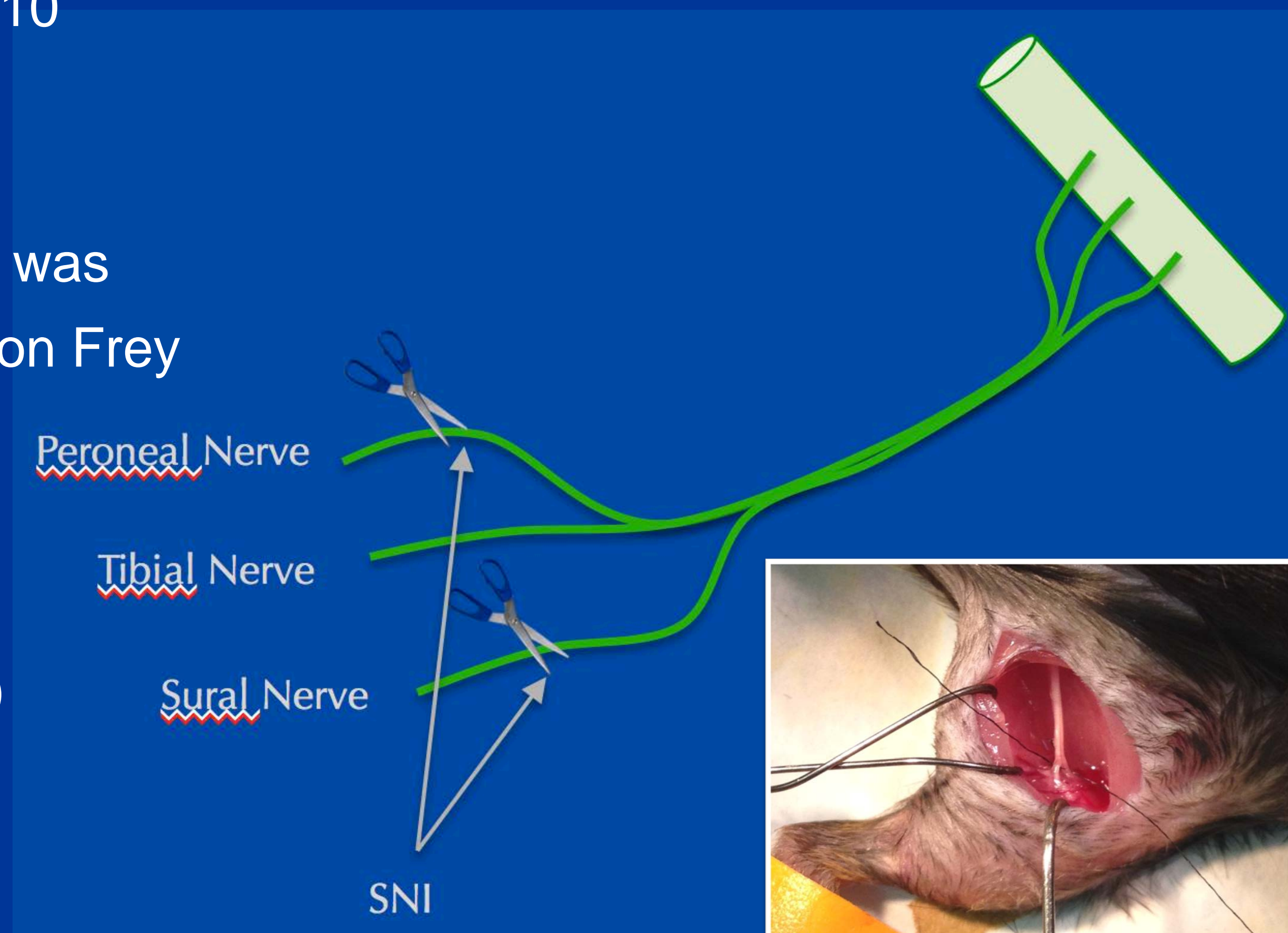
- Neuropathic pain is a common complication of nerve injury.
- Proposed mechanisms include NMDA receptor mediated central sensitization.
- It is unclear whether a preoperative subanesthetic dose of ketamine can prevent chronic pain

Hypothesis

- We hypothesized that ketamine would attenuate the development of neuropathic pain behavior in a mouse model.

Methods

- After IACUC approval, 30 C57/Bl6 mice were randomly allocated into three groups:
 - Spared nerve injury (SNI), N=10
 - Sham surgery, N=10
 - Ketamine/SNI, N=10
- Mechanical withdrawal threshold was measured using an electronic von Frey anesthesiometer.



Results

- The SNI group showed a statistically significant ($P < 0.01$) decrease in paw withdrawal threshold (PWT) on the surgical hind paw compared with sham.
- Compared to the SNI group, the treatment group demonstrated statistically significant ($P < 0.01$) increases in PWT.

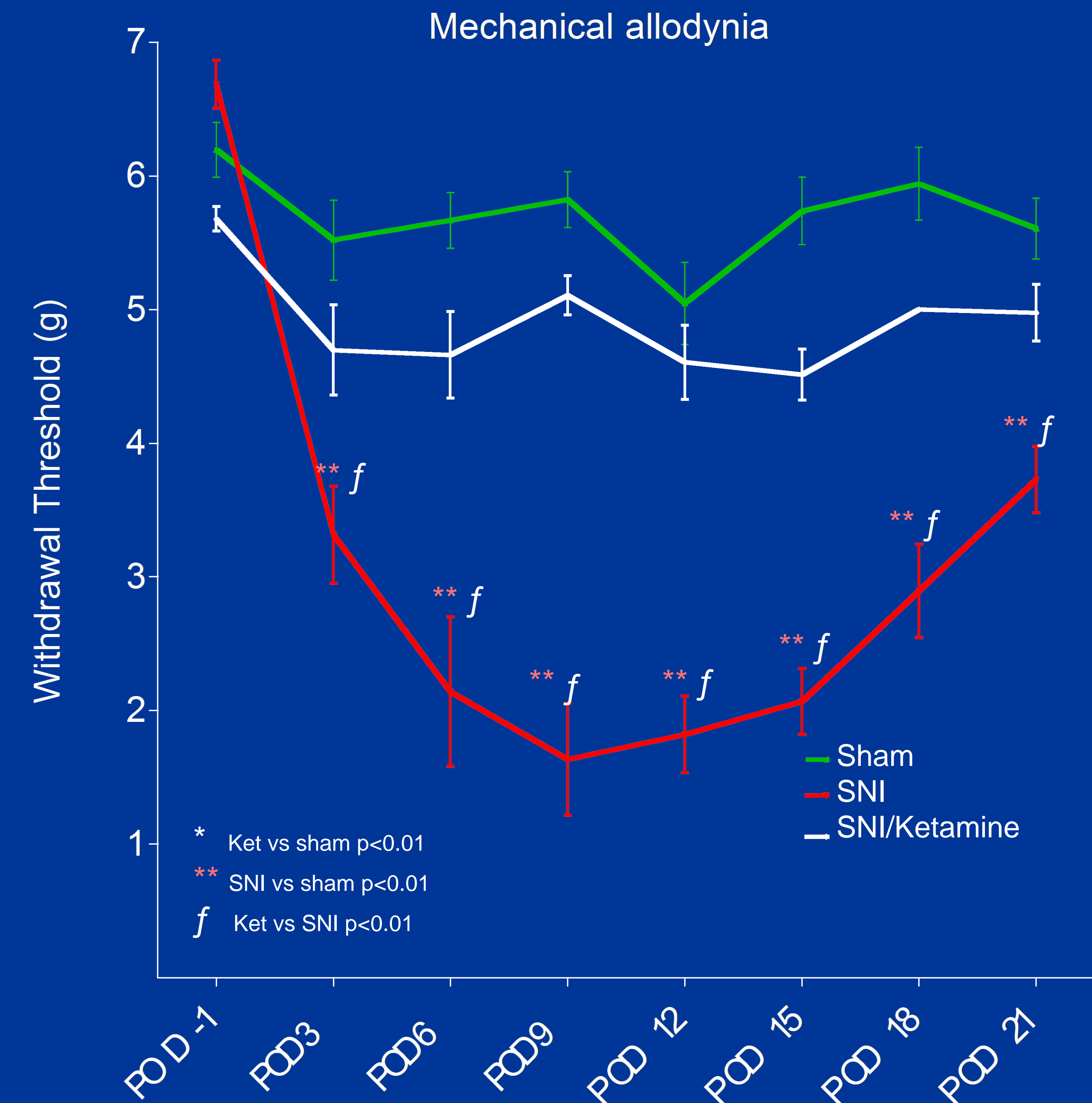


Figure: Withdrawal threshold vs post-operative day. Analysis by repeated measures ANOVA with Tukey post-hoc analysis.

Conclusions

- A one-time, preoperative, subanesthetic dose of ketamine attenuates neuropathic pain behavior.

References

1. Shields S, Eckert W, Basbaum A. The Journal of Pain, Vol. 4, No 8: pp 465-470
2. Mogil J, Graham A, Ritchie J, Hughes S, Austin J, Schorscher-PetCu A, Bennett G. Molecular Pain 2010, 6:34.

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Supported by NIH T32 #2T32GM008600-16 and DMRDP Grant #DM102142



Interspecies Plasma Metabolomics - Candidate Pain Pathway Prioritization

Hung-Lun John Hsia MD, Thomas VandeVen MD PhD, Thomas Buchheit MD, Joseph Lucas PhD, Mary McDuffie RN, Chester Buckenmaier MD, and Andrew Shaw MB FRCA

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Background:

Persistent pain after surgical nerve damage is a significant problem, affecting patients undergoing many different procedures. The biological pathways responsible are poorly characterized, and little progress has been made in the field of novel analgesic development. In order to prioritize the biological pathways of relevance we have compared the plasma metabolomes of humans with and without persistent pain after surgical amputation and C57/BI6 mice undergoing spared nerve injury. We hypothesize that pathways that are demonstrably important in both species represent high priority candidates for further mechanistic study and therapeutic target discovery.

Methods:

After IACUC approval, 30 C57/BI6 mice were randomly allocated into three groups:

- Sham surgery, N=5
- Spared Nerve Injury (SNI), N=15
- Dexamethasone/SNI, N=5

Observation of greatest phenotypic difference (paw withdrawal threshold levels) occurred on POD 15. At that time plasma was drawn from all mice and flash frozen at -80C and sent off for metabolic analysis.

After IRB approval and acquired consent, fifteen patients were selected from the Veterans Investigative Pain Evaluation Research (VIPER) cohort group and allocated to two groups based on a formal adjudication process to differentiate clinical pain phenotypes. This process placed them into two distinct groups:

- Control group, N=9
- Case group, N=6

Both groups received surgical amputations. The case group consisted of patients with the most severe pain scores. In contrast, the control group exhibited the lowest pain scores.

Results:

In mouse, 583 metabolites were analyzed and quantified, consisting of 345 named and 238 unnamed biochemicals. In humans, 658 metabolites, consisting of 363 named and 295 unnamed biochemicals, were analyzed. Plot scores demonstrate clear metabolic profile separation between groups in both human and mouse.

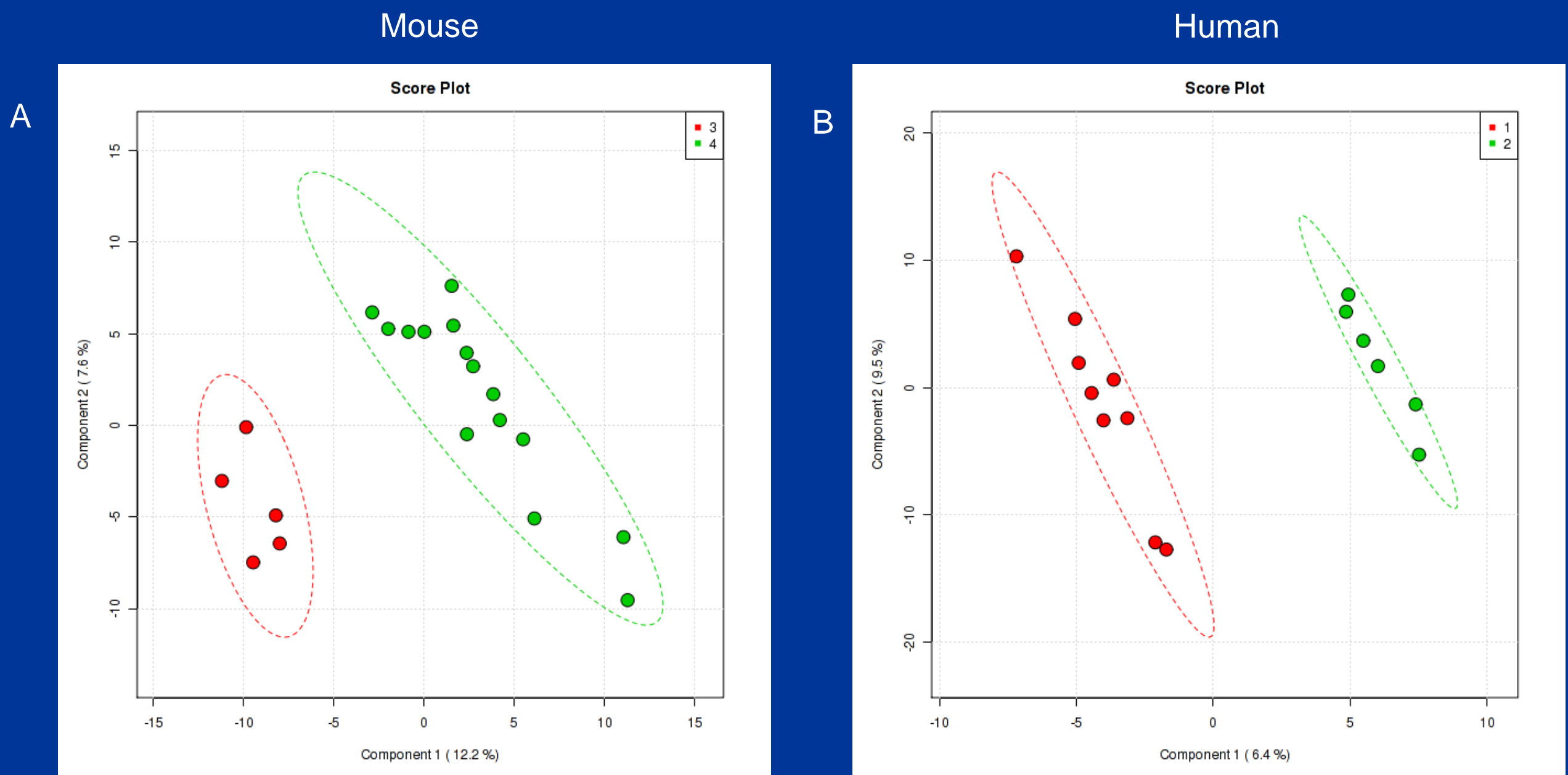


Fig 1: The figures above are score plots demonstrating the separation of differential metabolic profiles between groups in both mouse and humans. (A) Mouse sham surgery group (red dots) separated from nerve ligation group (green dots). (B) Human control group (red dots) separated from case group (green dots)

Statistical Comparisons Mouse Plasma				
ANOVA Contrasts	Total number of biochemicals with $p \leq 0.05$	Biochemicals ($\uparrow \downarrow$) $p \leq 0.05$	Total number of biochemicals with $0.05 < p < 0.10$	Biochemicals ($\uparrow \downarrow$) $0.05 < p < 0.10$
INT CTRL	51	11 40	40	13 27
TRT CTRL	183	75 108	34	16 18
TRT INT	177	93 84	51	21 30
One Way ANOVA	Total number of biochemicals with $p \leq 0.05$		Total number of biochemicals with $0.05 < p < 0.10$	
Group Effect	184		43	

Table 1: One way ANOVA analysis demonstrating significant differences in biochemical species between groups

Welch's Two Sample t-Test Human Plasma	CASE CTRL
Total number of biochemicals with $p \leq 0.05$	18
Biochemicals ($\uparrow \downarrow$)	7 11
Total number of biochemicals with $0.05 < p < 0.10$	27
Biochemicals ($\uparrow \downarrow$)	10 17

Table 2: Welch's Two Sample t-Test demonstrating significant differences between the two human groups

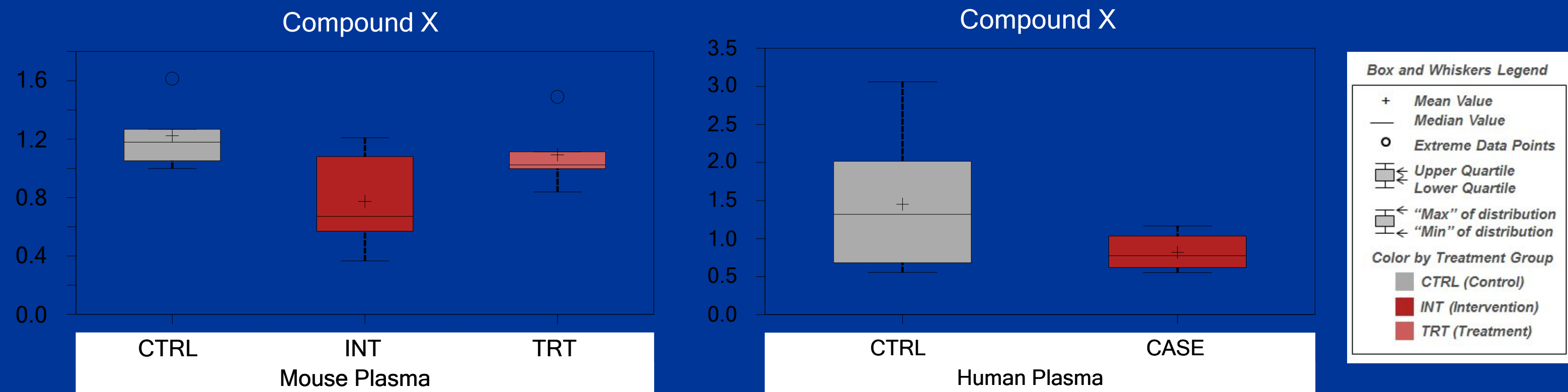


Fig 2: The graphs above demonstrate an identical biochemical species in both mouse and human which is down-regulated in nerve injury pain

Conclusions:

There were significant differences in the metabolic profiles between groups in both mouse and humans. Also, there is preserved cross-species differential expression of specific metabolic products.

References:

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Veterans Integrated Pain Evaluation Research (VIPER): Post-Amputation Pain Phenotypes in Injured Military Service Personnel

Thomas Buchheit MD, Thomas Van de Ven MD PhD, David MacLeod, MB FRCA, Mary McDuffie RN, Hung-Lun John Hsia MD, COL Chester “Trip” Buckenmaier MD, and Andrew Shaw MB FRCA

Departments of Anesthesiology, Duke University Medical Center, Walter Reed National Military Medical Center, and Durham Veterans Affairs Medical Center



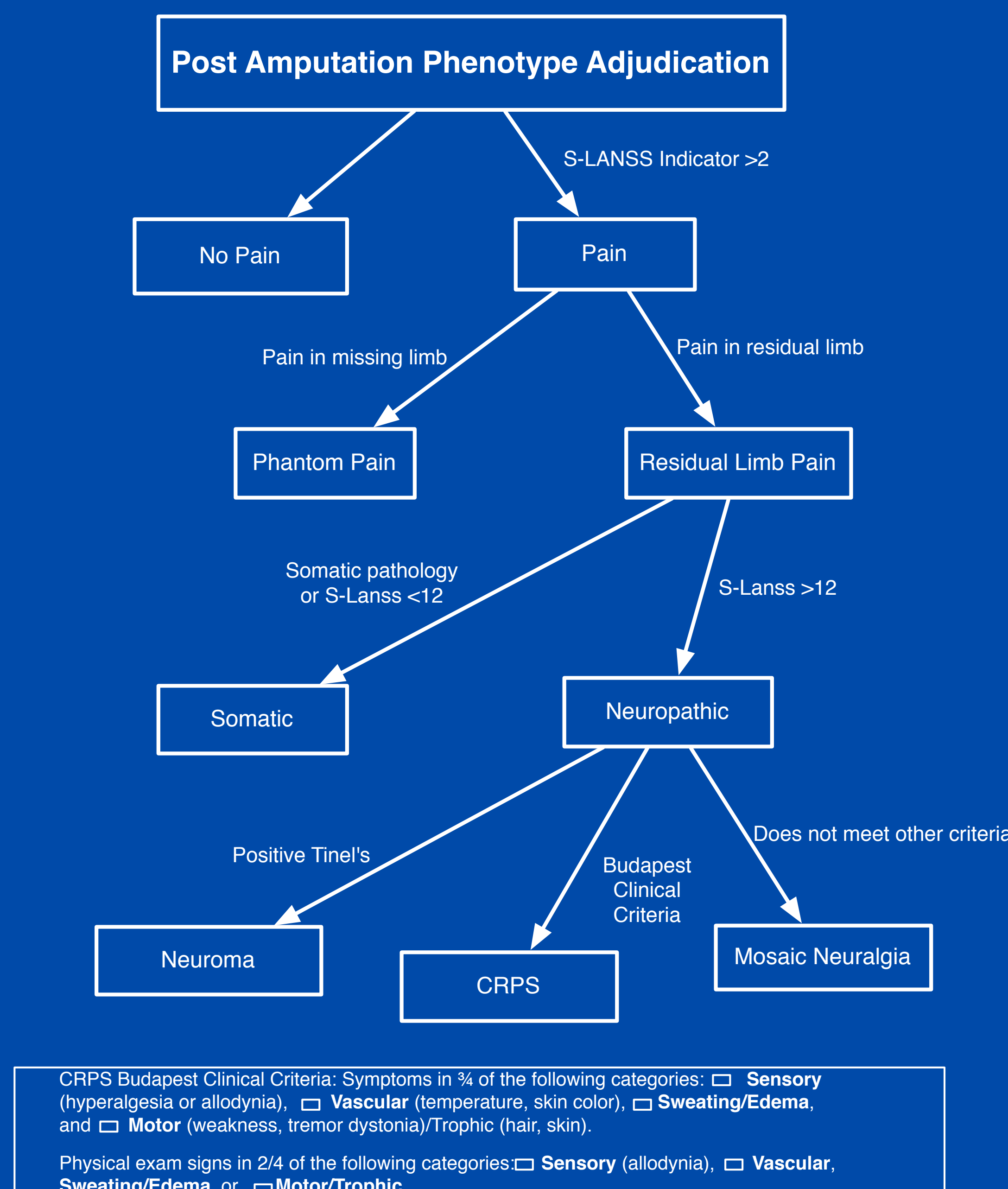
Background

- Chronic pain is a common problem in injured military service members undergoing amputation.¹
- Most studies of post-amputation pain only discriminate phantom and residual limb pain.²
- Sub-classification of pain phenotypes is a likely important step in the development of disease-specific therapies.
- A collaborative study (Veterans Integrated Pain Evaluation Research (VIPER)) between Duke University, Walter Reed National Military Medical Center (WRNMMC) and the Durham VAMC is being conducted to further define post-amputation clinical phenotypes and to correlate these findings with circulating biomarkers of persistent pain.
- Here we report on the initial cohort of 41 military service members who have undergone clinical assessment and phenotypic adjudication.

Methods Phenotypic Assignment

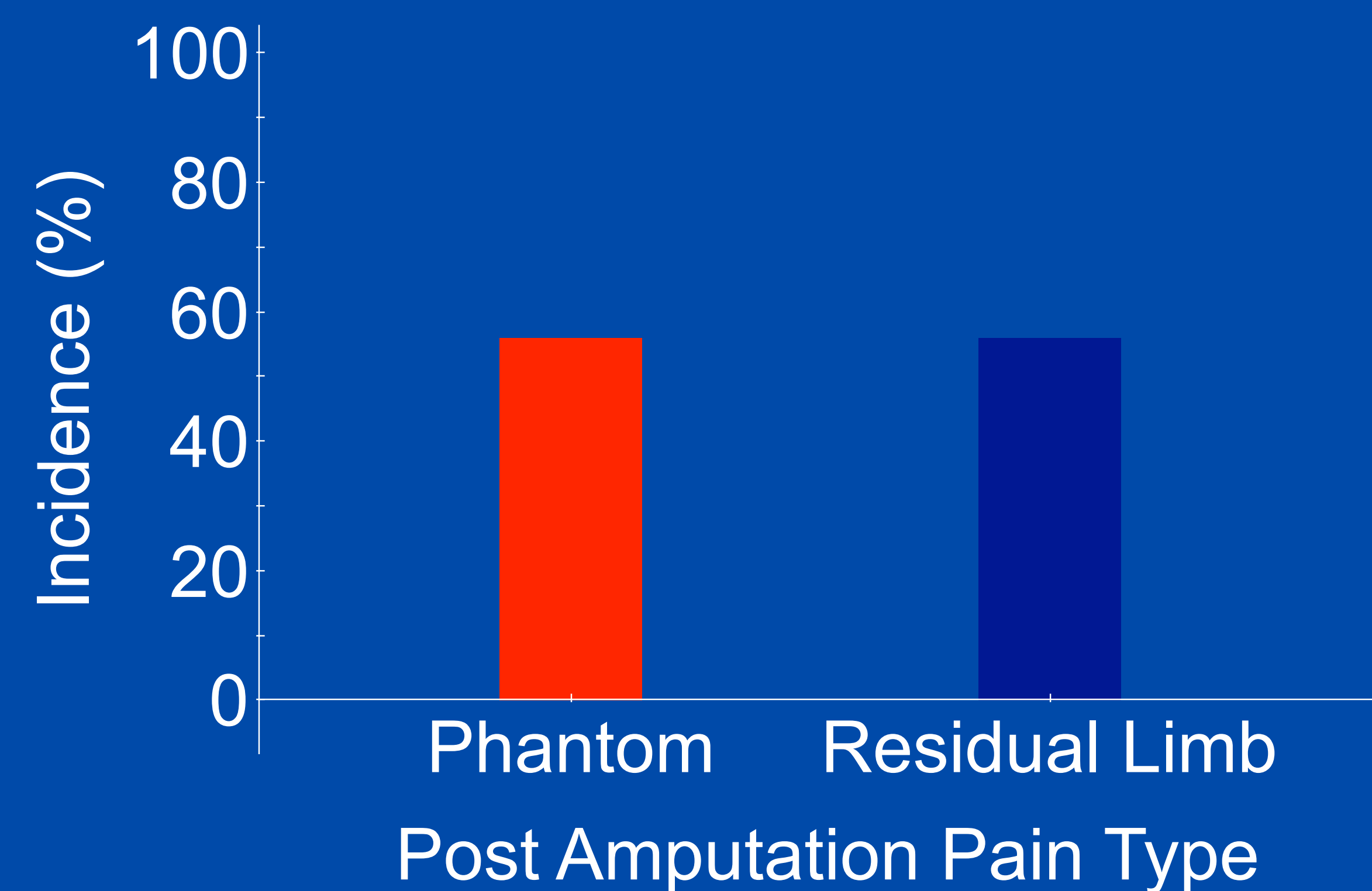
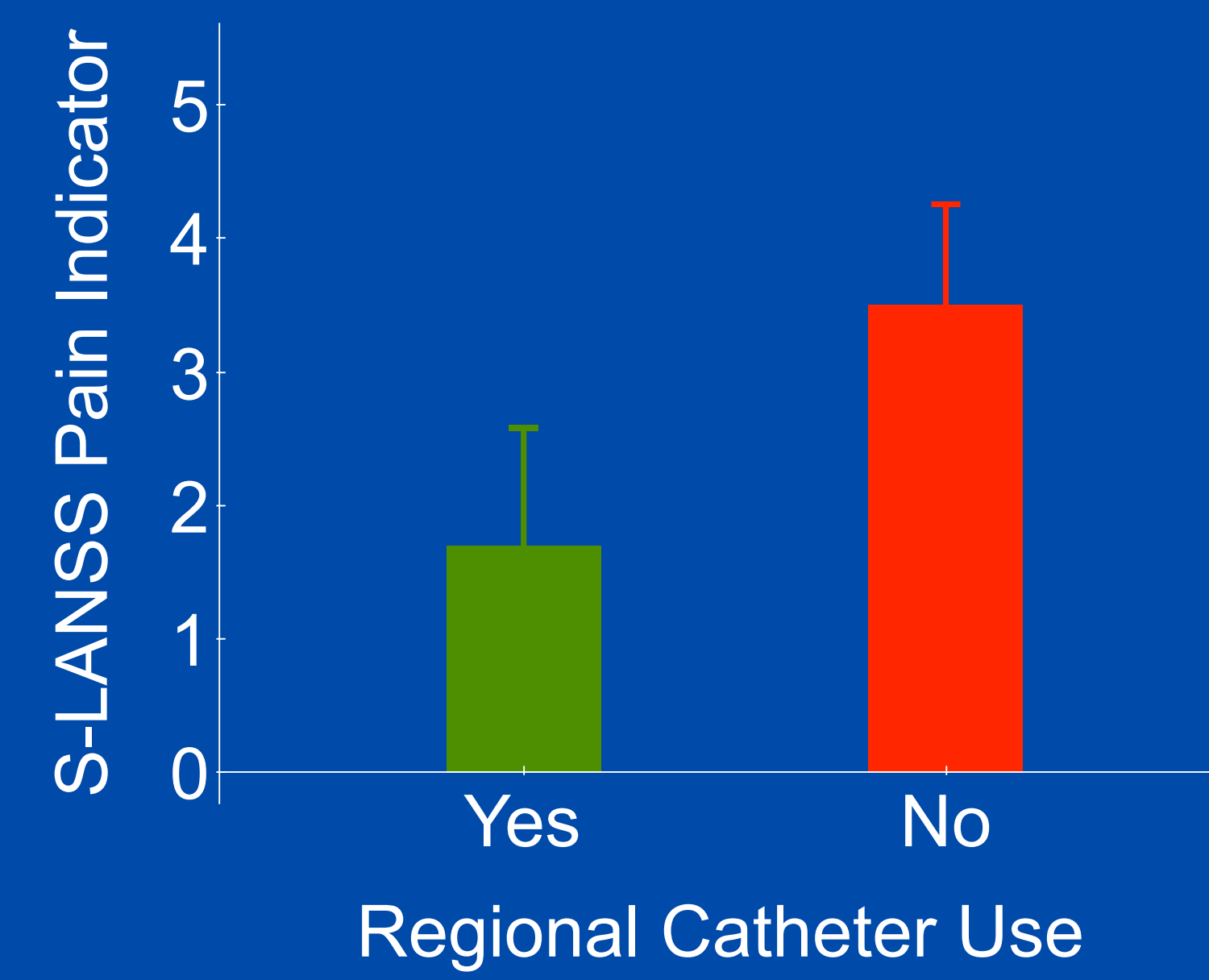
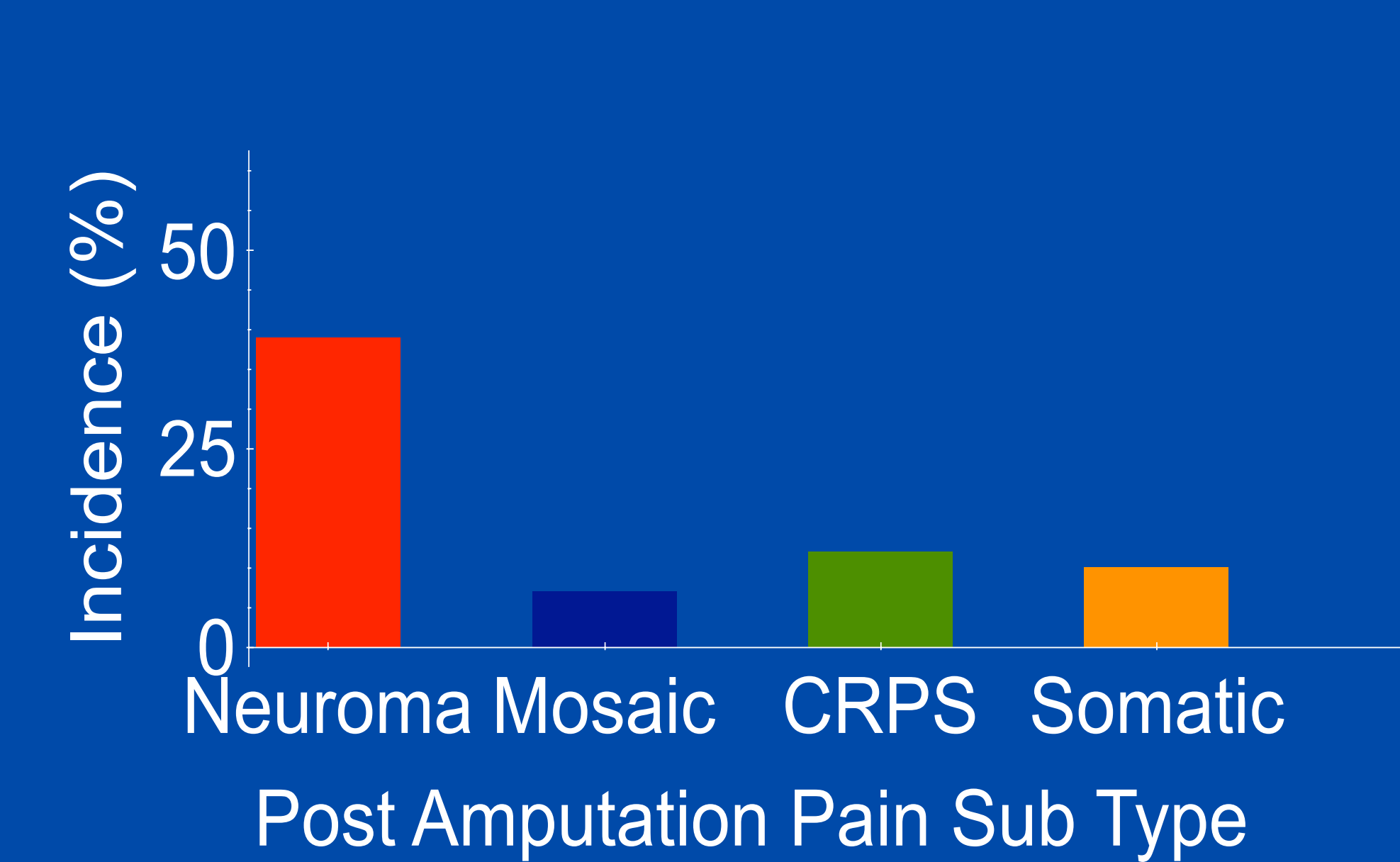
After IRB approval, the VIPER clinical cohort was assessed using validated questionnaire instruments:

- Brief Pain Inventory (BPI)
- Self-Reported Leeds Assessment of Neuropathic Symptoms and Signs Pain Scale (S-LANSS)
- Complex Regional Pain Syndrome (Budapest Clinical Criteria)
- Phantom and residual limb pain questionnaires
- A formal endpoint adjudication was performed using the algorithm previously reported by our group³
 - Phantom and residual limb pain were discriminated.
 - Residual limb pain was then sub-categorized into a) Neuroma b) CRPS c) Mosaic Neuralgia or d) Somatic.



Results

- Using the Duke Post-Amputation Pain Algorithm (Duke PAPA), we discriminated between several post-amputation pain subtypes in this preliminary cohort of military service members.
- We found an overall incidence of post-amputation pain of 61%.
 - 56% described phantom pain
 - 56% described residual limb pain (RLP)
 - There was significant overlap with these diagnoses, but they did not always co-exist
- Of those subjects with RLP the following diagnostic categories were noted:
 - 70% neuroma
 - 22% CRPS
 - 13% Mosaic neuralgia (neuralgic pain not otherwise specified)
 - 17% somatic
- We additionally observed that the use of regional anesthesia catheters at the time of injury is associated with a decreased incidence of post-amputation pain during our assessment.
 - This effect appears secondary to reductions in residual limb pain, but not reductions in phantom pain.



Conclusions

- We observed phenotypic complexity of post-amputation pain symptoms in this initial cohort including:
 - Significant but not complete overlap in the diagnoses of phantom and residual limb pain
 - Several distinct subtypes of residual limb neuropathic pain
 - A predominant contribution of neuroma symptoms in service members with residual limb pain
- We additionally observed that the use of regional anesthesia catheters at the time of injury is associated with a decreased incidence of chronic pain during our assessment.

References

- Reiber GE, McFarland LV, Hubbard S, et al. Servicemembers and veterans with major traumatic limb loss from Vietnam war and OIF/OEF conflicts: survey methods, participants, and summary findings. J Rehabil Res Dev. 2010;47(4):275-297.
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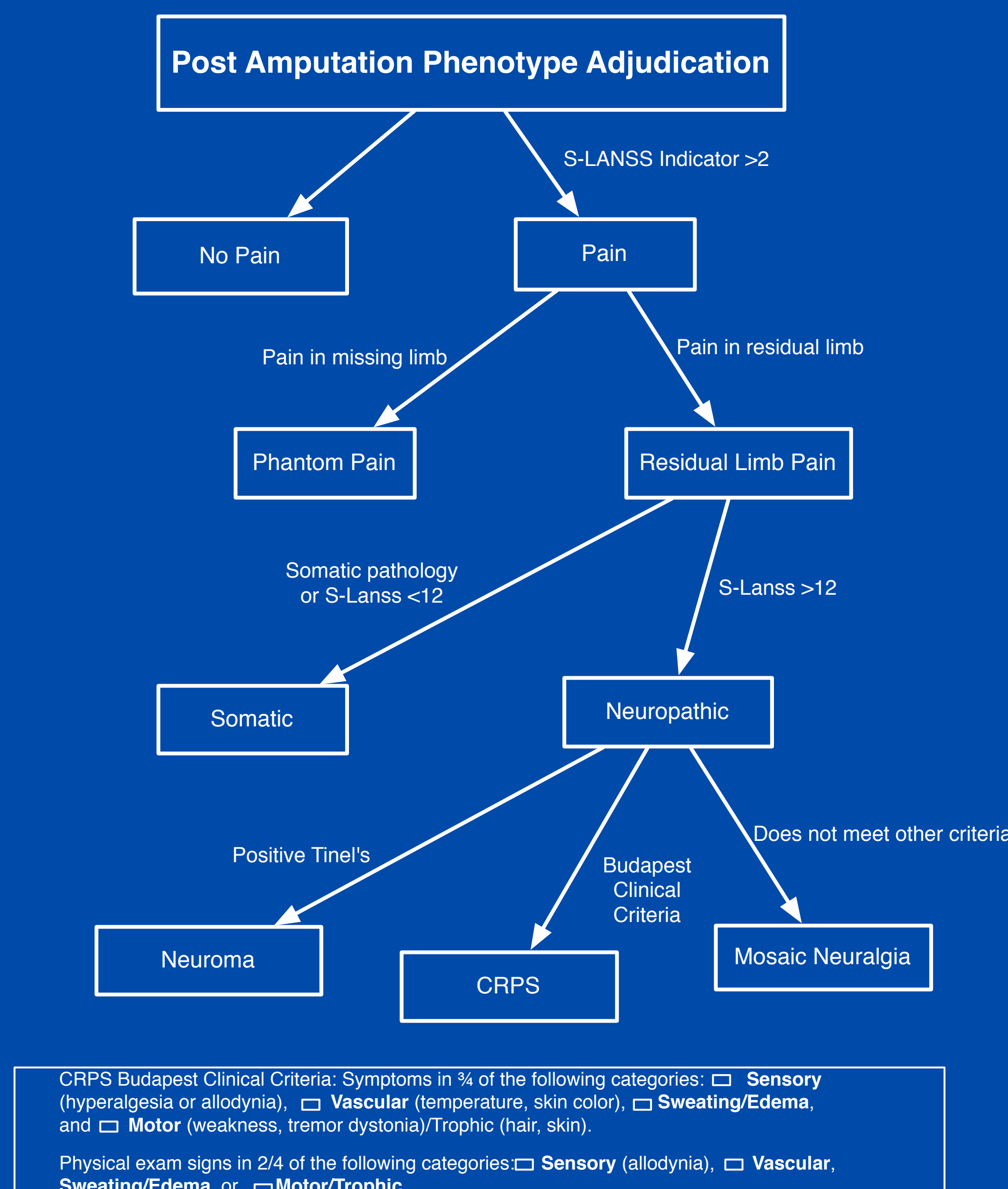
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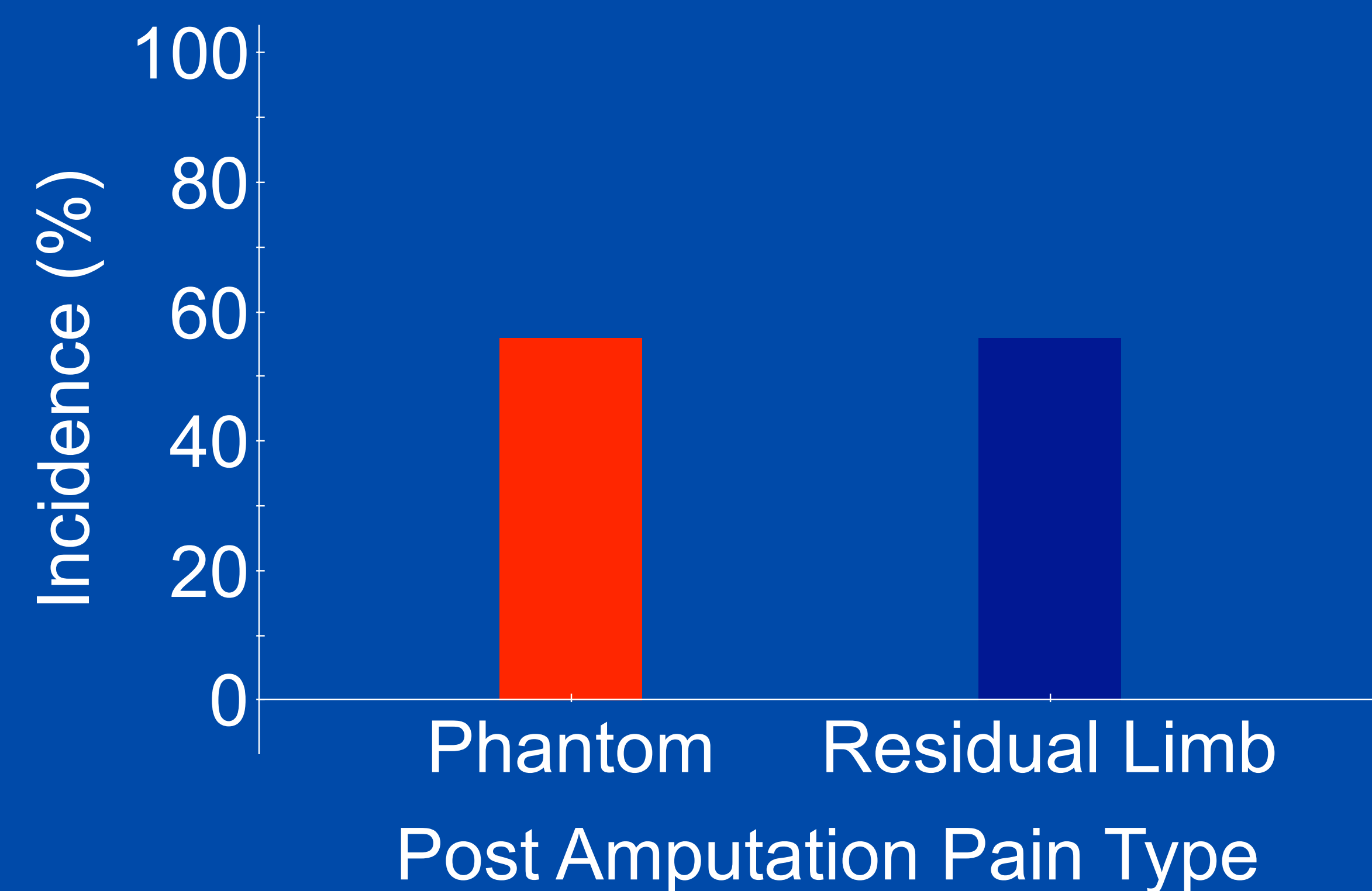
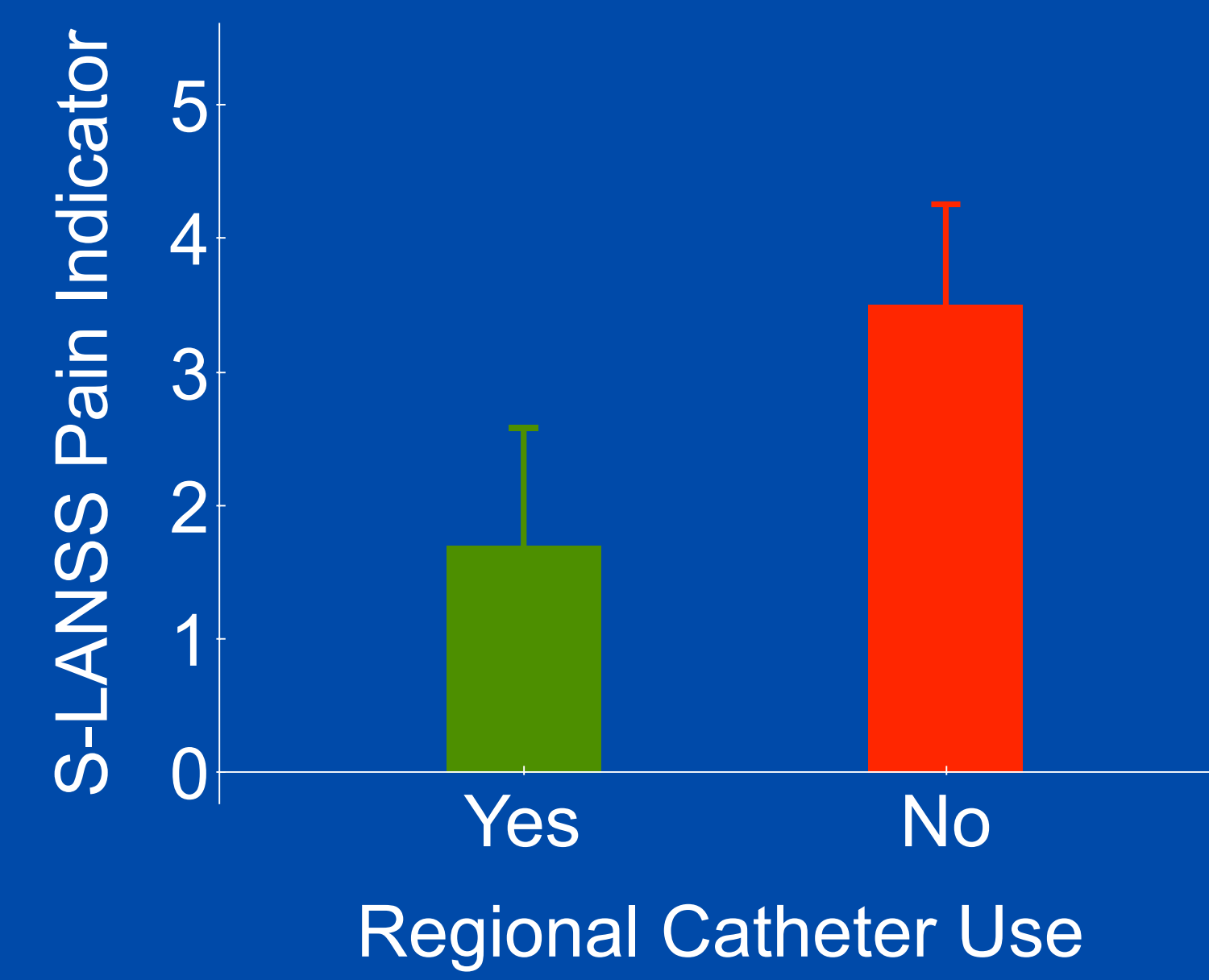
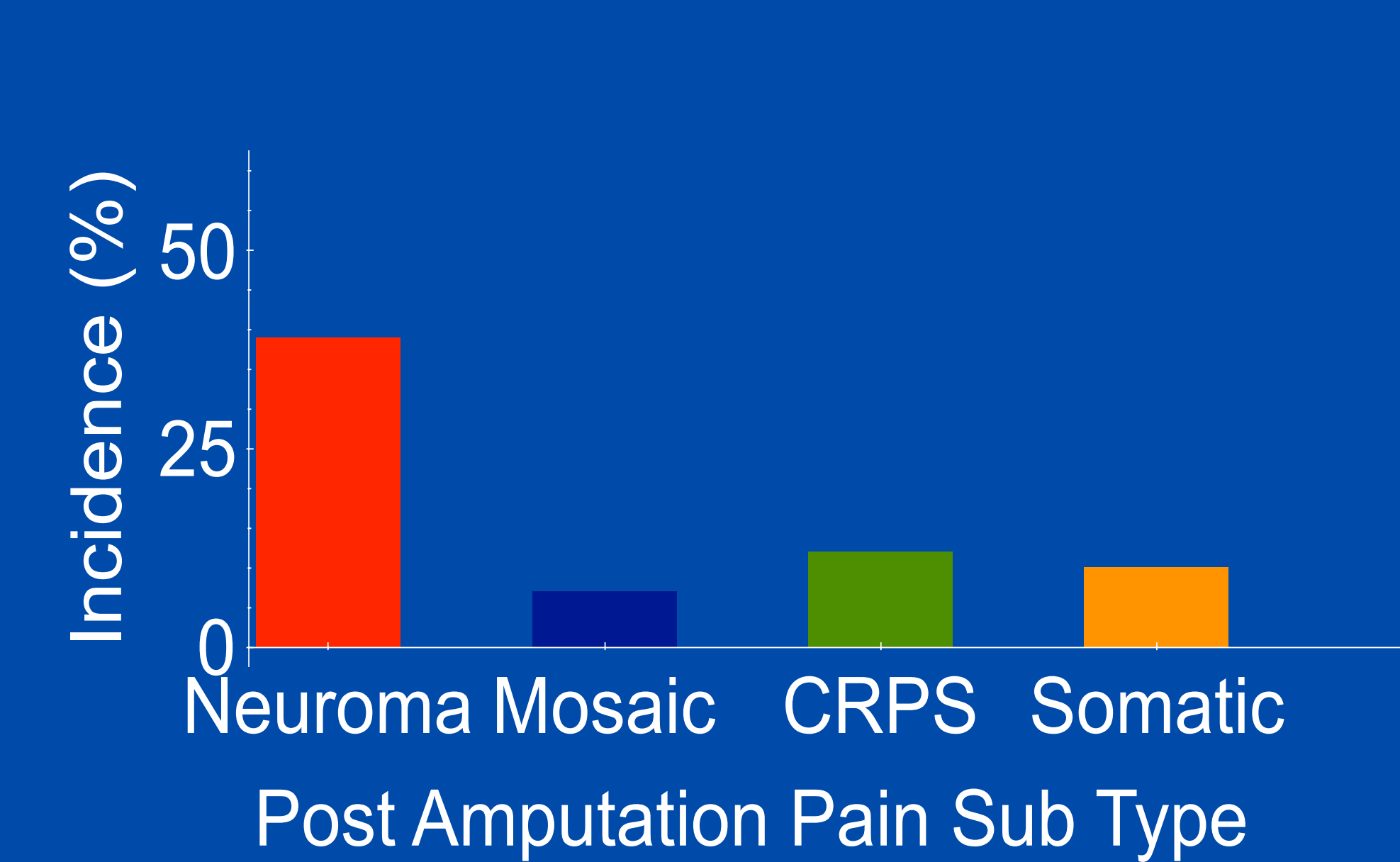
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Supported by DMRDP Grant #DM102142



Veterans Integrated Pain Evaluation Research (VIPER) Pilot Cohort: Feasibility Of Studying Combat Amputation Pain

Andrew Shaw MB FRCA, Tom Buchheit MD, Tom Vandeven MD PhD, David Macleod MB FRCA, Mary McDuffie RN, Nancy Kwon RN, John Hsia MD, Mary Kirkley, Trip Buckenmaier MD

Departments of Anesthesiology, Duke University Medical Center, Durham, NC; Walter Reed National Military Medical Center, Bethesda, MD; Durham VA Medical Center, Durham, NC



Introduction

- We are studying combat amputation injury in OIF/OEF/OND personnel
- Our overarching goal is to identify novel biomarkers of amputation pain subtypes and better define the mechanisms involved in the transition from acute to chronic nerve injury pain
- Here we report summary clinical data from our pilot cohort

Patients

- 41 subjects with traumatic amputation have been enrolled since January 2012
- All sustained a traumatic injury leading to loss of a limb
- Data are collected between 3 and 18 months after initial injury in theater

Endpoints

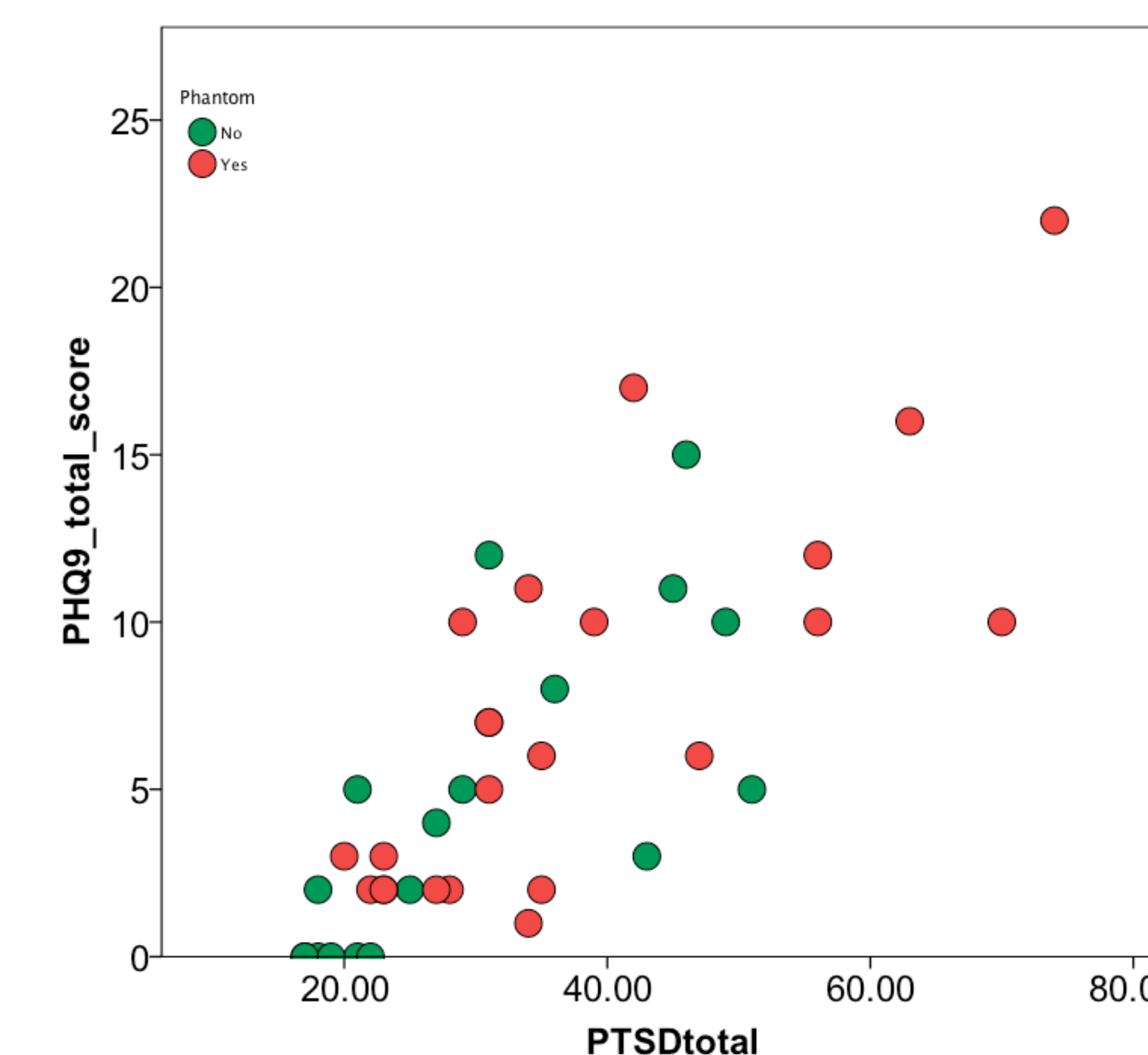
- We collect pain type and severity data using the following instruments:
- BPI, S-LANSS, PHQ 9, PCS, VAS, PTSD-M

Control = S LANSS <3
Case = S LANSS >2

Total N = 41	Controls (mean)	Cases (mean)	P value
Regional anesthesia catheter used (%)	31.2	16	NS
Ever smoker (%)	81.2	32	<0.05
Age	25	26	NS
BMI	25.7	26.6	NS
S-LANSS total	10	14	<0.05
S-LANSS indicator	0.88	4.48	<0.001
VAS (0-100)	4	24	0.001
PTSD total	28.9	37.8	<0.05
BPI worst	1	4	<0.05
BPI interfere	0.5	2.3	<0.05
PCS total	3	10	0.01
Stump pain (%)	0	56	<0.001
Phantom pain (%)	0	56	<0.001

Results

- There is a clear relationship between perceived impact of pain on quality of life and severity of PTSD symptoms
- This is independent of whether the pain is residual limb type or phantom



Comment

- This study, and the newly funded follow on intervention study, will provide detailed data regarding the epidemiology and molecular characteristics of the different subtypes of post amputation pain in military service personnel.



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Veterans Integrated Pain Evaluation Research (VIPER) Pilot Cohort: Feasibility Of Studying Combat Amputation Pain

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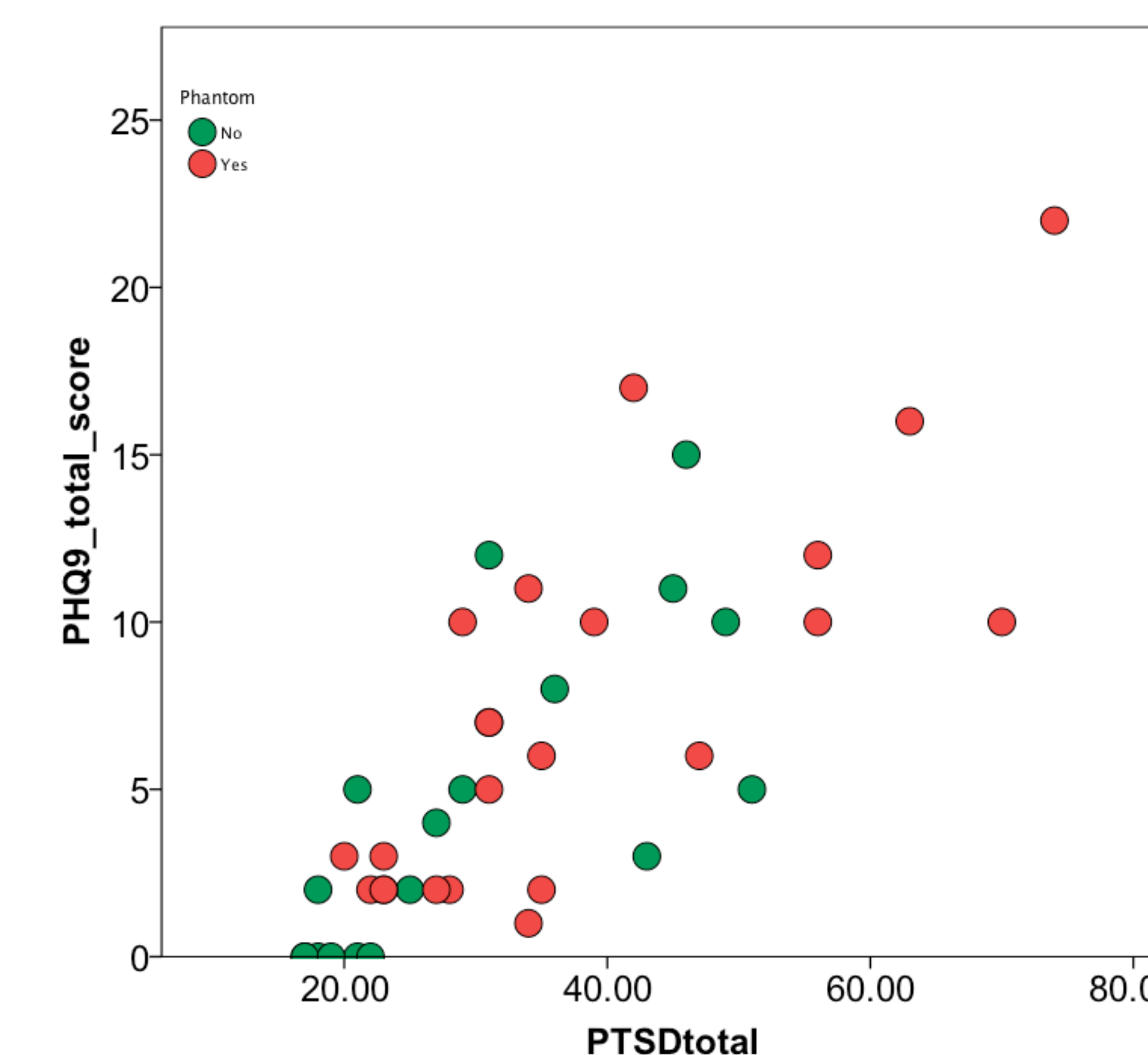
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Supported by DMRDP Grant #DM102142



Department of Anesthesiology, Duke University Medical Center, Walter Reed National Military Medical Center, Durham Veterans Affairs Medical Center

- Functional annotation and pathway mapping was performed to find pathways with multiple differentially methylated components between amputees with and without pain.

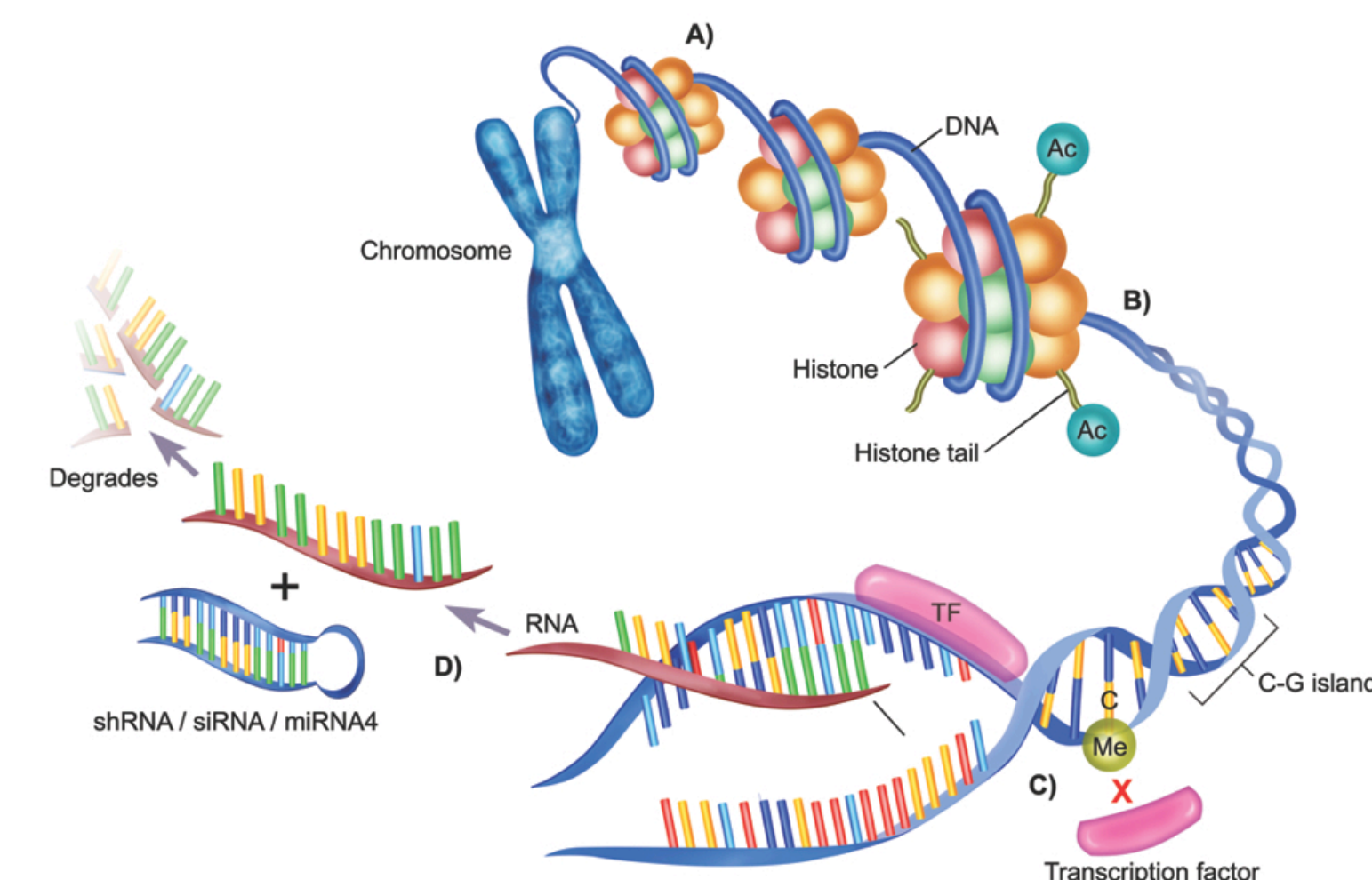


Figure 1: Epigenetic modifications include histone acetylation, DNA methylation and miRNA dependent RNA degradation

- Blood was collected into PAXgene RNA and PAXgene DNA tubes and DNA and RNA were extracted using Qiagen PAXgene RNA and DNA extraction kits.

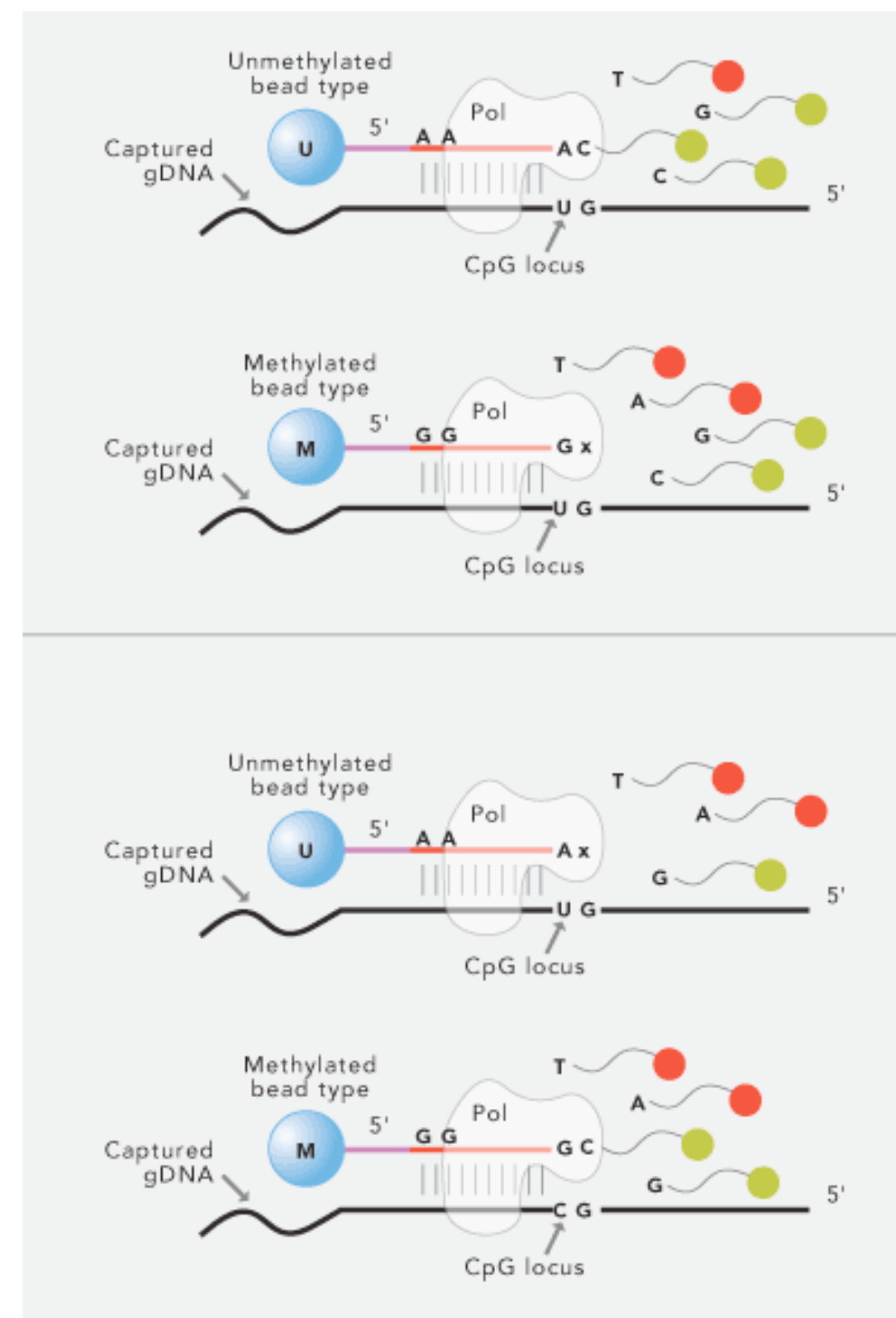


Figure 2: Illumina Infinium 450K DNA methylation array. Methylated cytosine does not become thymine after bisulfite conversion.

- Data analysis was performed using the Illumina's Genome Studio software and the R-based methylation array analysis module MethLAB¹.

- Pathway discovery was performed using the DAVID functional annotation tool².

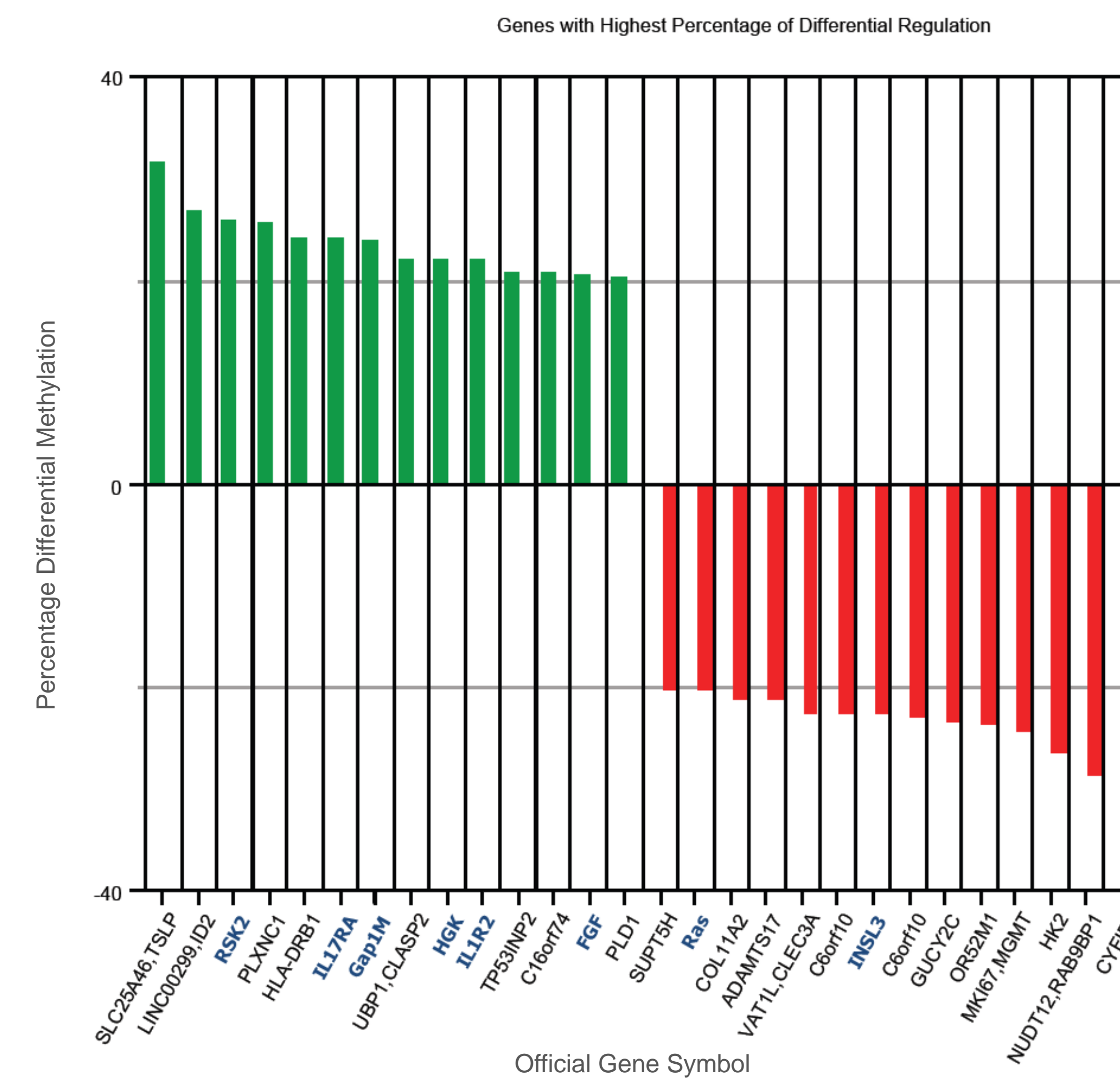


Figure 2: Gene promoter regions most significantly differentially methylated between amputees with and without pain (MAPK pathway members highlighted in blue)

- After QC/normalization and $\Delta\beta$ determination using Illumina Genome Studio software, 81 genes with promoter regions showing 20% or greater differential methylation between groups were identified. The most significant genes (by p-value) are listed in Figure 2. Results were confirmed using the Methlab R-based module.

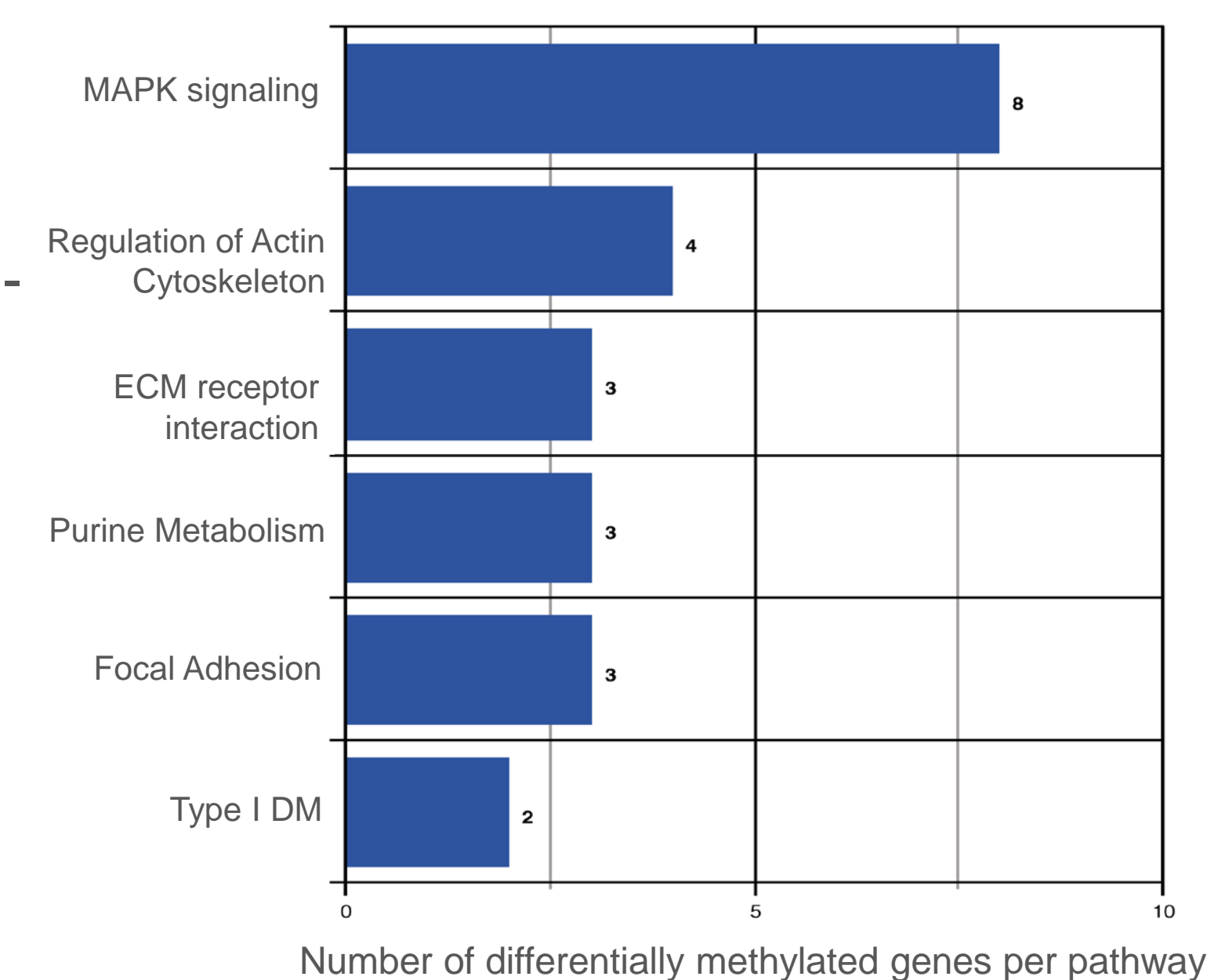


Figure 3: Most highly represented pathways from the list of differentially methylated genes between groups

- The eight genes differentially methylated between groups are shown within the KEGG³ derived MAPK pathway (Figure 4).

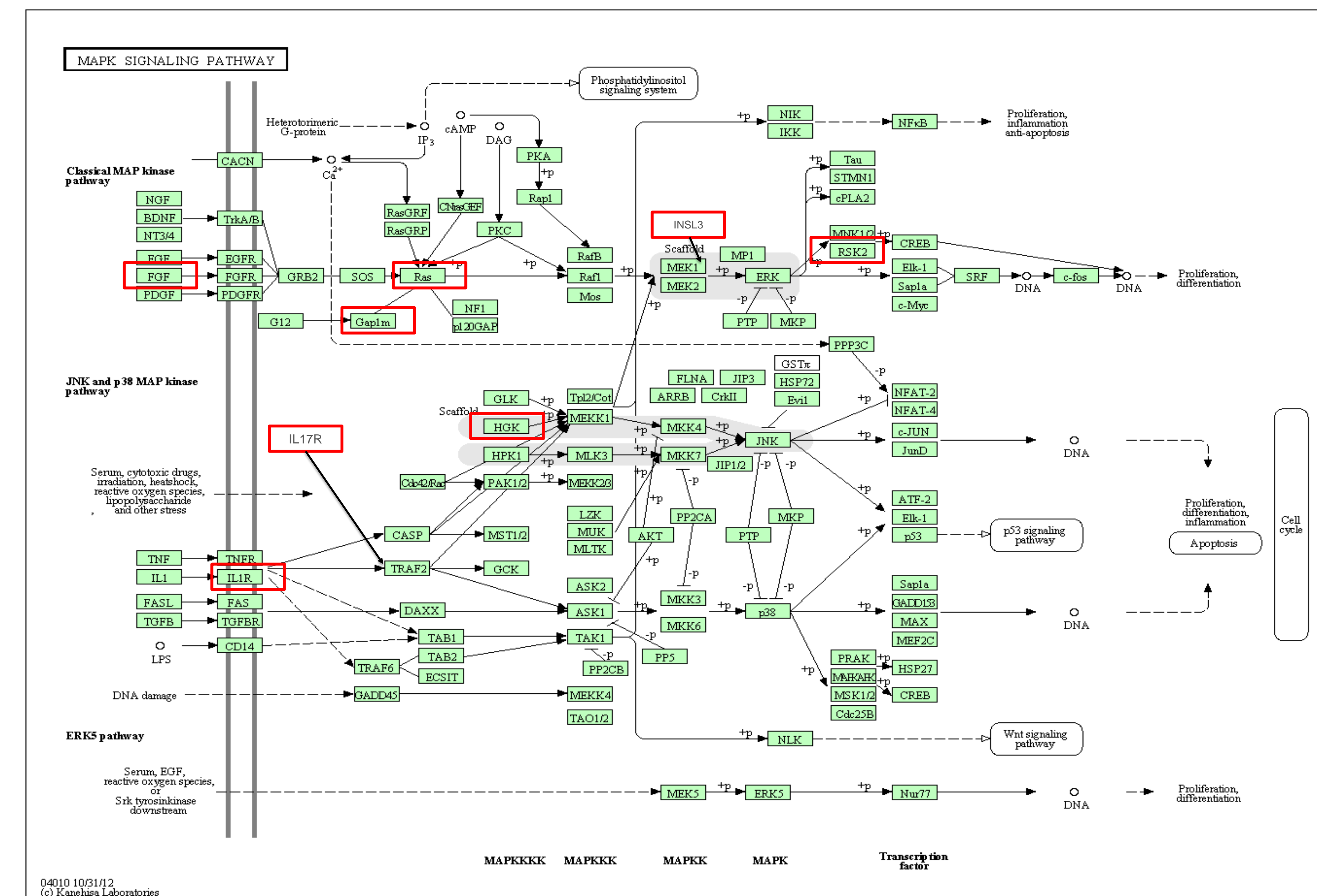


Figure 4: Differentially methylated genes and their position in the MAPK pathway (KEGG)

- The MAPK pathway is well-known as an important regulator of the transition from acute to chronic pain⁴.
- Soldier amputees with and without chronic pain 3-18 months after surgery or trauma show significant differences in gene promoter methylation of multiple MAPK pathway genes.
- Gene expression analysis is ongoing and will allow correlation between methylation and expression for each of these genes of interest.
- The timing of this differential methylation is unknown but may be clarified by the VIPER Valproate study that includes collection of DNA samples before and after amputation.

- 1. MethLAB software – Killaru et al. *Epigenetics*. 2012 Mar;7(3):225-9.
- 2. DAVID - <http://david.abcc.ncifcrf.gov/summary.jsp>
- 3. KEGG MAPK pathway - <http://www.genome.jp/kegg/pathway/hsa/hsa04010.html>
- 4. Ji, RR, Suter, MR. *Molecular Pain* 2007, 3:33

Grant support: 1.) DOD CDMRP DM102142 VIPER: Veteran's Integrated Pain Evaluation Research
2.) NIH T32 T32 2T32GM008600-16 Integrated Training in Anesthesiology Research



Regional Anesthesia Catheters Reduce the Incidence of Neuropathic Post-Amputation Pain: Results from the VIPER Cohort of Injured Military Personnel

Hung-Lun J. Hsia MD, Thomas Buchheit MD, Thomas Van de Ven MD PhD, David MacLeod, MB FRCA, Mary McDuffie RN, William White MS, COL Chester “Trip” Buckenmaier MD, and Andrew Shaw MB FRCA

Departments of Anesthesiology, Duke University Medical Center, Walter Reed National Military Medical Center, and Durham Veterans Affairs Medical Center



Background

- Chronic pain is a common problem in injured military service members undergoing amputation.¹
- Most studies of post-amputation pain only discriminate phantom and residual limb pain.²
- Sub-classification of pain phenotypes is an important step in the development of disease-specific therapies.
- An ongoing collaborative study (Veterans Integrated Pain Evaluation Research (VIPER)) between Duke University, Walter Reed National Military Medical Center (WRNMMC) and the Durham VAMC is being conducted to further define post-amputation clinical phenotypes and discover circulating biomarkers of persistent pain.
- Here we make a report on 124 military service members who have undergone clinical assessment and phenotypic adjudication.

Methods

Phenotypic Assignment

After IRB approval, the VIPER clinical cohort was assessed using validated questionnaire instruments:

- Brief Pain Inventory (BPI)
- Self-Reported Leeds Assessment of Neuropathic Symptoms and Signs Pain Scale (S-LANSS)
- Complex Regional Pain Syndrome (Budapest Clinical Criteria)
- Phantom and residual limb pain questionnaires
- A formal endpoint adjudication was performed using the algorithm previously reported by our group³
 - Phantom and residual limb pain were discriminated.
 - Residual limb pain was then sub-categorized into a) Neuroma b) CRPS c) Mosaic Neuralgia or d) Somatic.

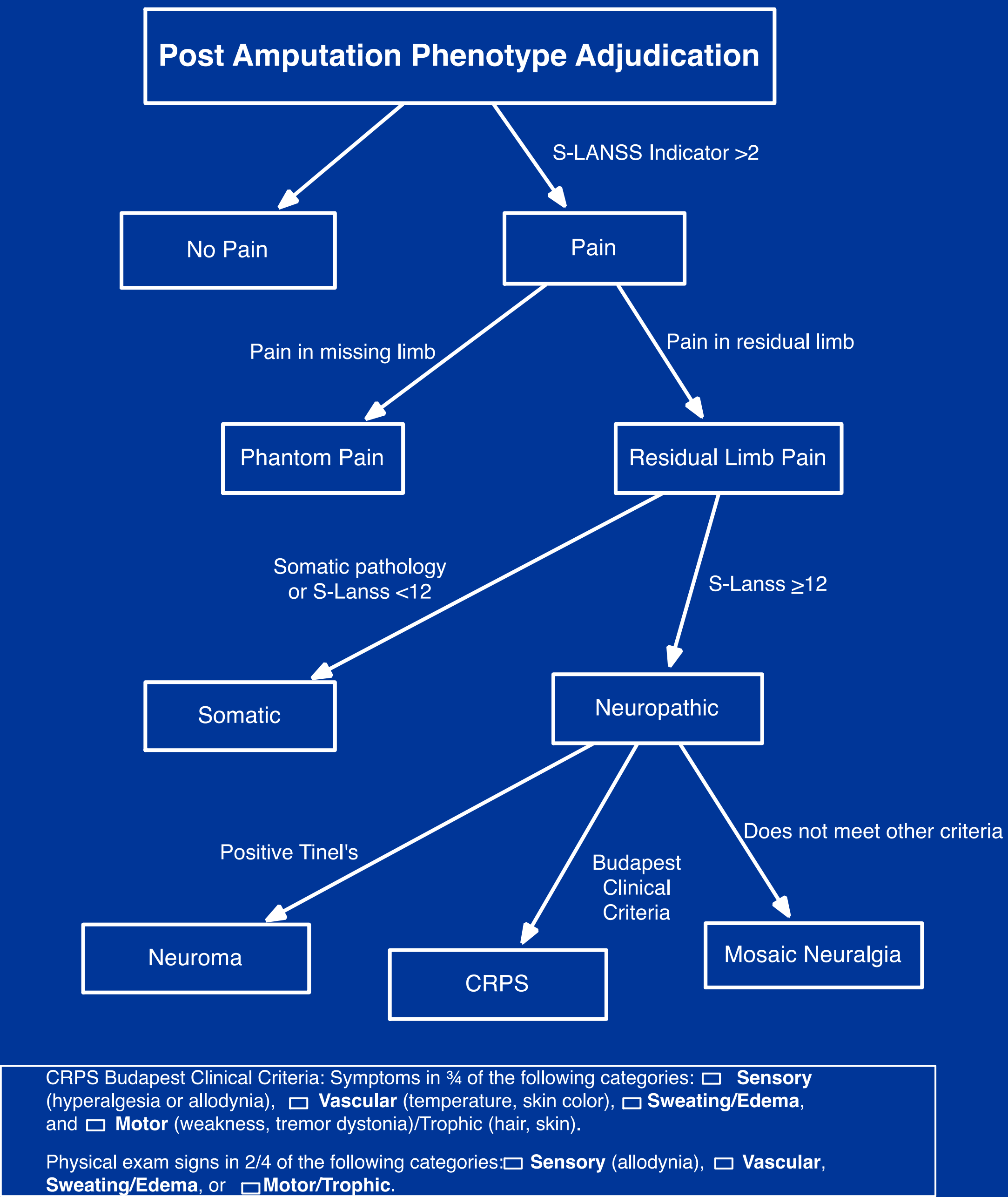


Figure 1: Adjudication algorithm utilized to assign patients to their respective groups.

Results

- Using the Duke Post-Amputation Pain Algorithm (Duke PAPA), we discriminated between several post-amputation pain subtypes in military service members.
 - We found an overall incidence of post-amputation pain (PAP) of 64.5%.
 - When these PAP cases were further sub-categorized:
 - 90% described phantom pain
 - 95% described residual limb pain (RLP)
 - There was significant overlap with these diagnoses, but they did not always co-exist
- Furthermore, of those subjects with RLP the following diagnostic categories were noted:
 - 46.3% neuroma
 - 18.8% CRPS
 - 10% Mosaic neuralgia (neuralgic pain not otherwise specified)
 - 38.8% somatic
- In our analysis of retrospective catheter placement data, we found a significantly decreased incidence of neuropathic pain in patients receiving regional catheters within 7 days of injury.

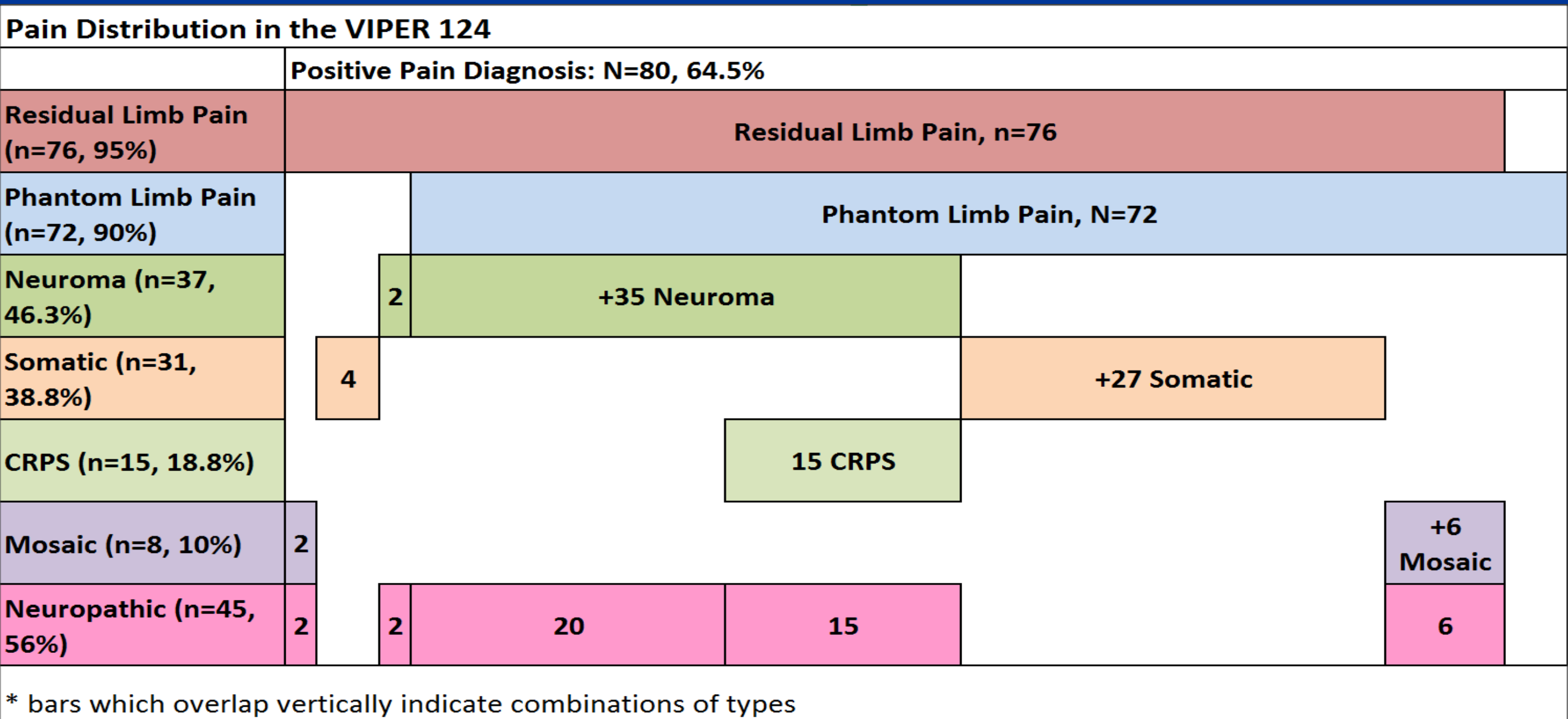


Figure 2: Phenotypic variation in post-amputation pain demonstrating significant overlap between residual limb pain and phantom limb pain. Also, there is substantial overlap between RLP-Neuroma and RLP-CRPS

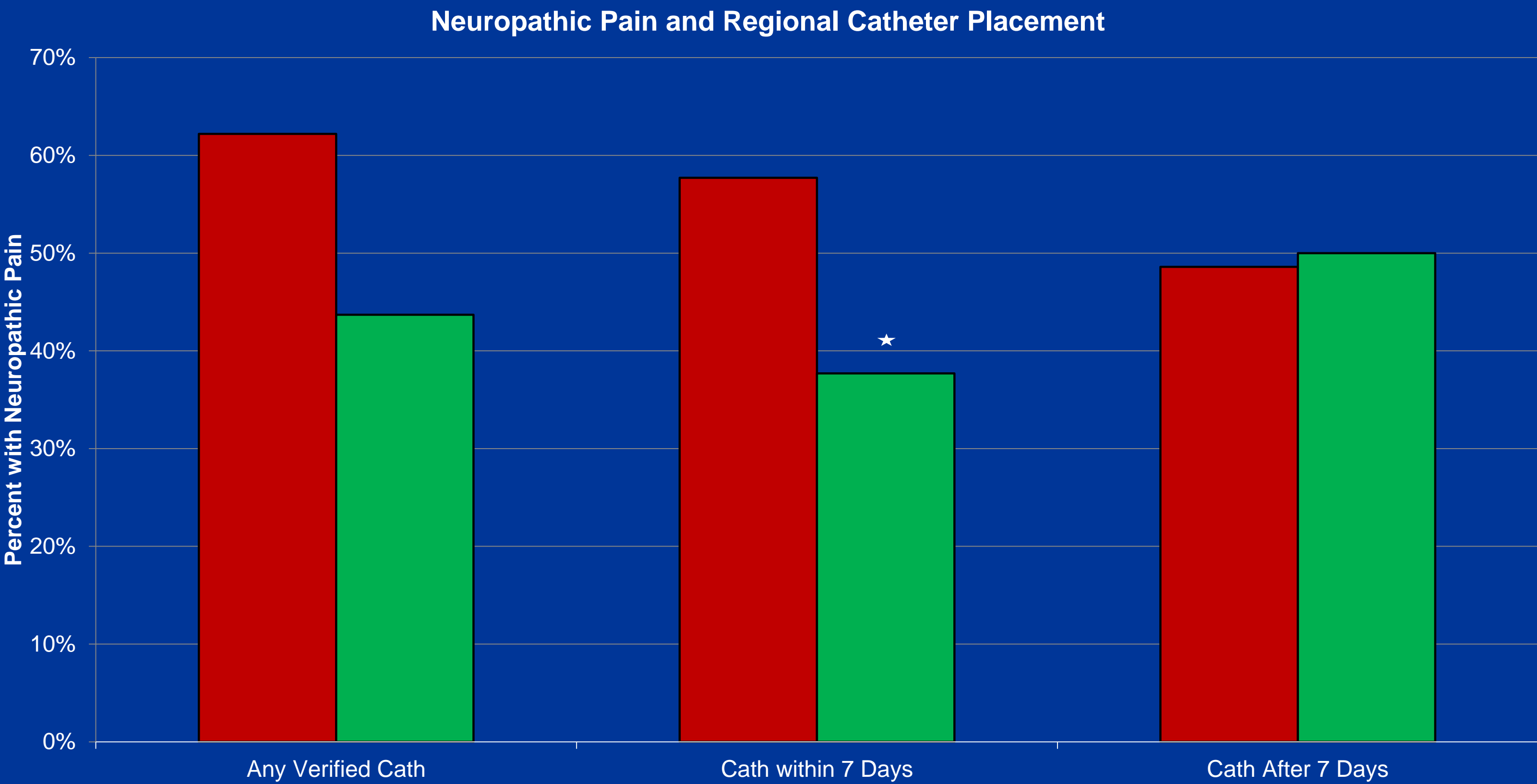


Figure 3: Regional Catheter use and decreased incidence of pain with varying timeframes of placement. The * denotes statistical significance.

Conclusions

- We observed phenotypic complexity of post-amputation pain symptoms in this cohort including:
 - Strong overlap in the diagnoses of phantom and residual limb pain
 - Several distinct subtypes of residual limb neuropathic pain
 - A predominant contribution of neuroma symptoms in service members with residual limb pain
- Additionally, we observed that the use of early regional anesthesia catheters was associated with a decreased incidence of chronic neuropathic pain.

References

- Reiber GE, McFarland LV, Hubbard S, et al. Servicemembers and veterans with major traumatic limb loss from Vietnam war and OIF/OEF conflicts: survey methods, participants, and summary findings. J Rehabil Res Dev. 2010;47(4):275-297.
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Supported by Department of Defense (DM102142)

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More than Mere Detergents: An Interspecies Study Reveals Bile Acids as Novel Mediators in Acute and Chronic Pain

Alexander G. Chameessian BS^{1,5,6}, BS, Alex Kieber BS¹, Hung-Lun Hsia MD^{1,3}, Thomas Buchheit MD^{1,3}, Mary McDuffie, RN², Chester Buckenmeier MD², Andrew Shaw MB FRCA^{1,3}, Ru-Rong Ji PhD^{1,4}, Thomas Van de Ven MD PhD^{1,3}

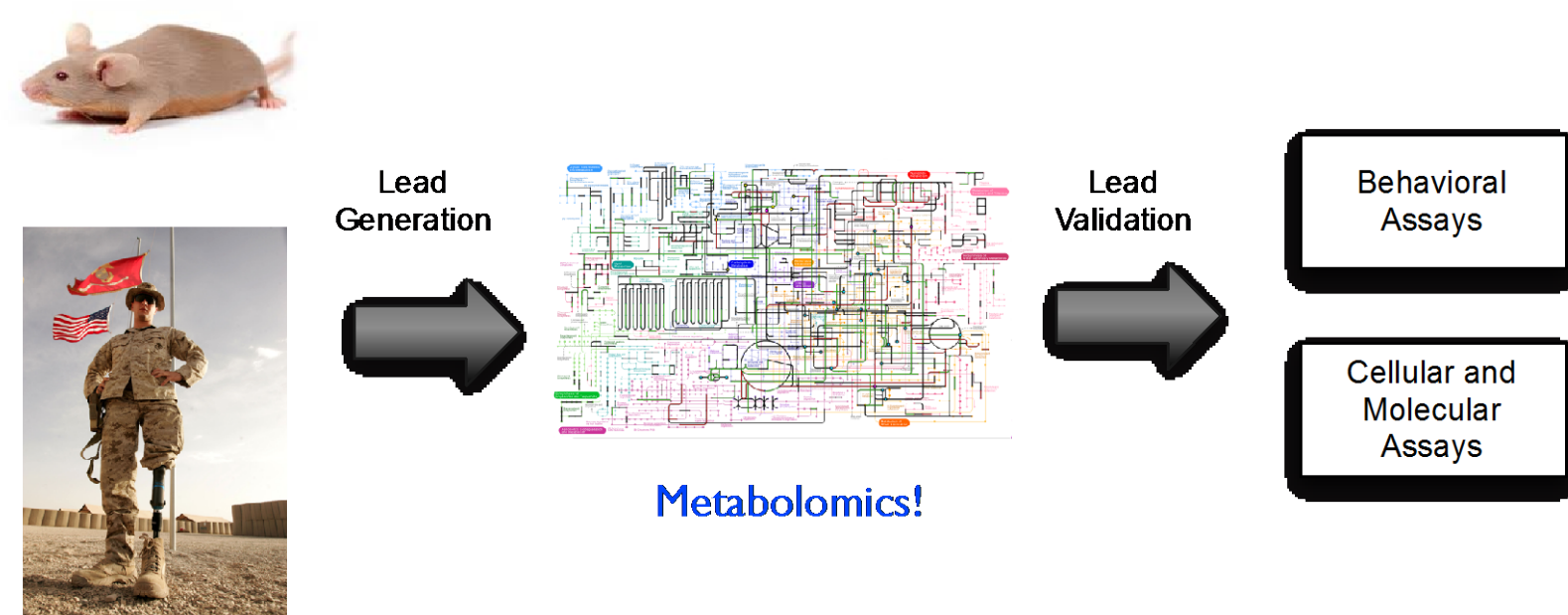


¹Department of Anesthesiology, Duke University Medical Center, Durham, NC USA, ²Walter Reed National Military Medical Center, Bethesda, MD USA, ³Durham Veterans Affairs Medical Center³, Durham, NC USA, ⁴Department of Neurobiology, Duke University, Durham, NC USA, ⁵Medical Scientist Training Program, Duke University, Durham, NC USA, ⁶Department of Pharmacology and Cancer Biology, Duke University, Durham, NC USA

Introduction

- Persistent pain after surgical nerve damage is a significant problem, affecting patients undergoing many different procedures¹. The biological pathways responsible are poorly characterized, and little progress has been made in the field of novel analgesic development.
- We initiated an exploratory, interspecies (human and mouse) investigation to identify novel pathways and mediators in the transition of acute to chronic pain.
- These exploratory efforts revealed several **bile acids** (deoxycholate, cholate) to be differentially regulated in pain and non-pain states in humans and mice

Materials and Methods



Metabolomics

- After IACUC approval, 30 C57/Bl6 mice were randomly allocated into three groups:
 - Sham surgery, N=5
 - Spared Nerve Injury (SNI), N=15
 - Dexamethasone/SNI, N=5
- At POD15 plasma was drawn from all mice and flash frozen at -80C and sent for metabolic analysis.
- Fifteen patients were selected from the Veterans Investigative Pain Evaluation Research (VIPER) cohort group and adjudicated into two groups representing extreme phenotypes.
 - Control, SLANSS <3
 - Case, SLANSS >2
- Data analysis performed using Metaboanalyst 2.0²

Behavioral Testing

- Male CD-1 mice were used for all behavioral testing
 - Naïve or Chronic Constriction Injury (CCI)
- Deoxycholate (DCA) was administered either via the intrathecal or intraplantar route
- Mechanical sensibility was assessed with von Frey filaments method (ref?) after treatment with DCA
- Acute, chemo-nociception was assayed using the capsaicin test after treatment with DCA
 - Intraplantar injection of 1 ng of capsaicin
 - Nocifensive behavior analyzed by video recording

Tissue Culture

- Astrocytes were harvested from p3 neonatal mice (C57/Bl6) and co-cultured with microglia for 3 weeks
- 3 days before testing with bile acid compounds, astrocytes were plated into 12-well plates and differentiated with cAMP (ref)
- Pre-treated for 1 hour with bile acid and then stimulated with the cytokine TNF-alpha (10 ng/ml)
- Supernatants collected and assayed for MCP-1 levels by ELISA

Results

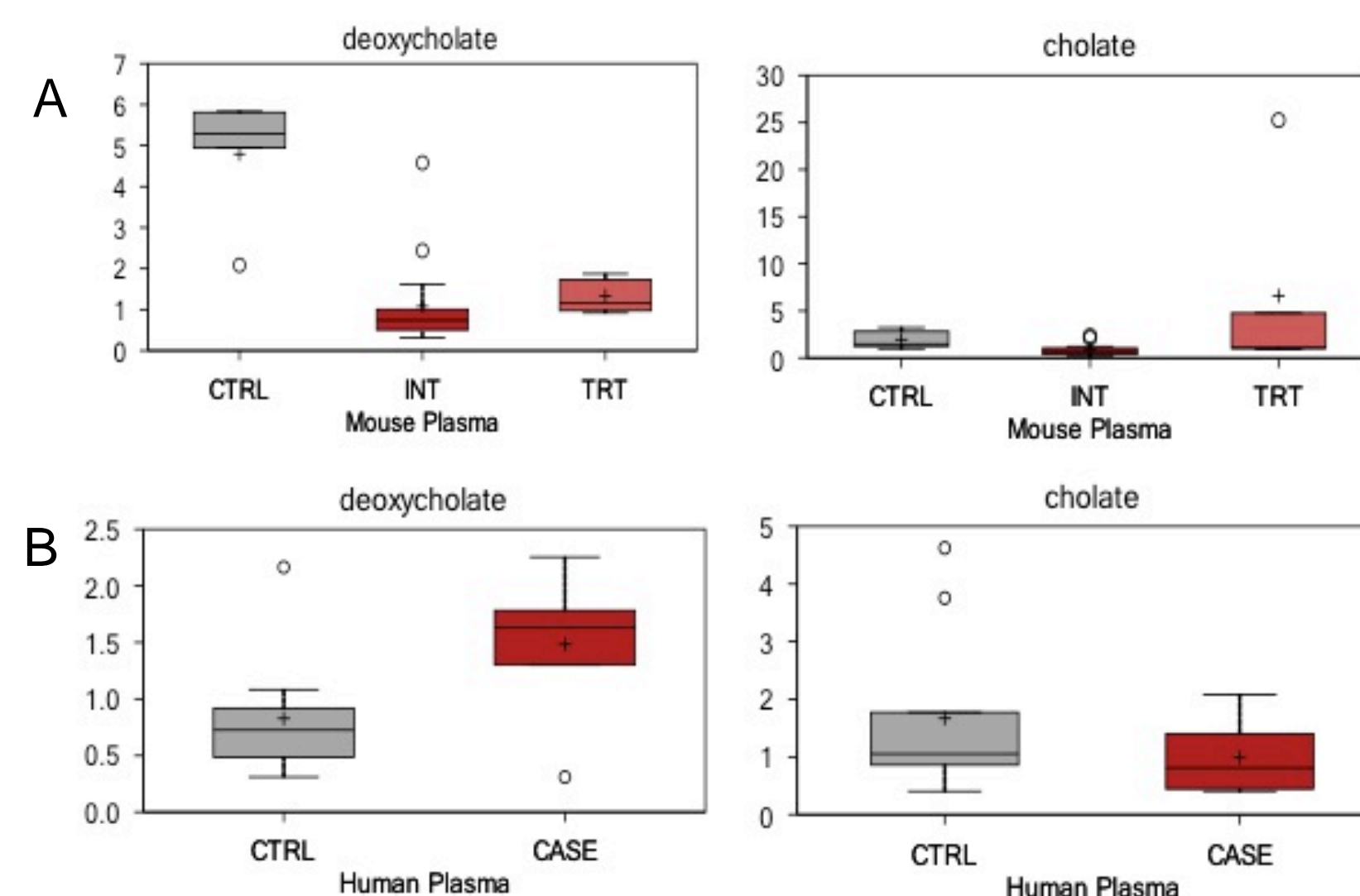


Figure 1. Metabolomic analysis of mouse and human plasma converges on the bile acid pathway. Deoxycholate and cholate differed significantly between pain and non-pain states in both mice and humans. (A) CTRL = Sham (N=5), INT = Spared Nerve Injury (SNI) (N=15), TRT = SNI/Dexamethasone (N=5). (B). CTRL = SLANSS < 3, CASE = SLANSS > 2

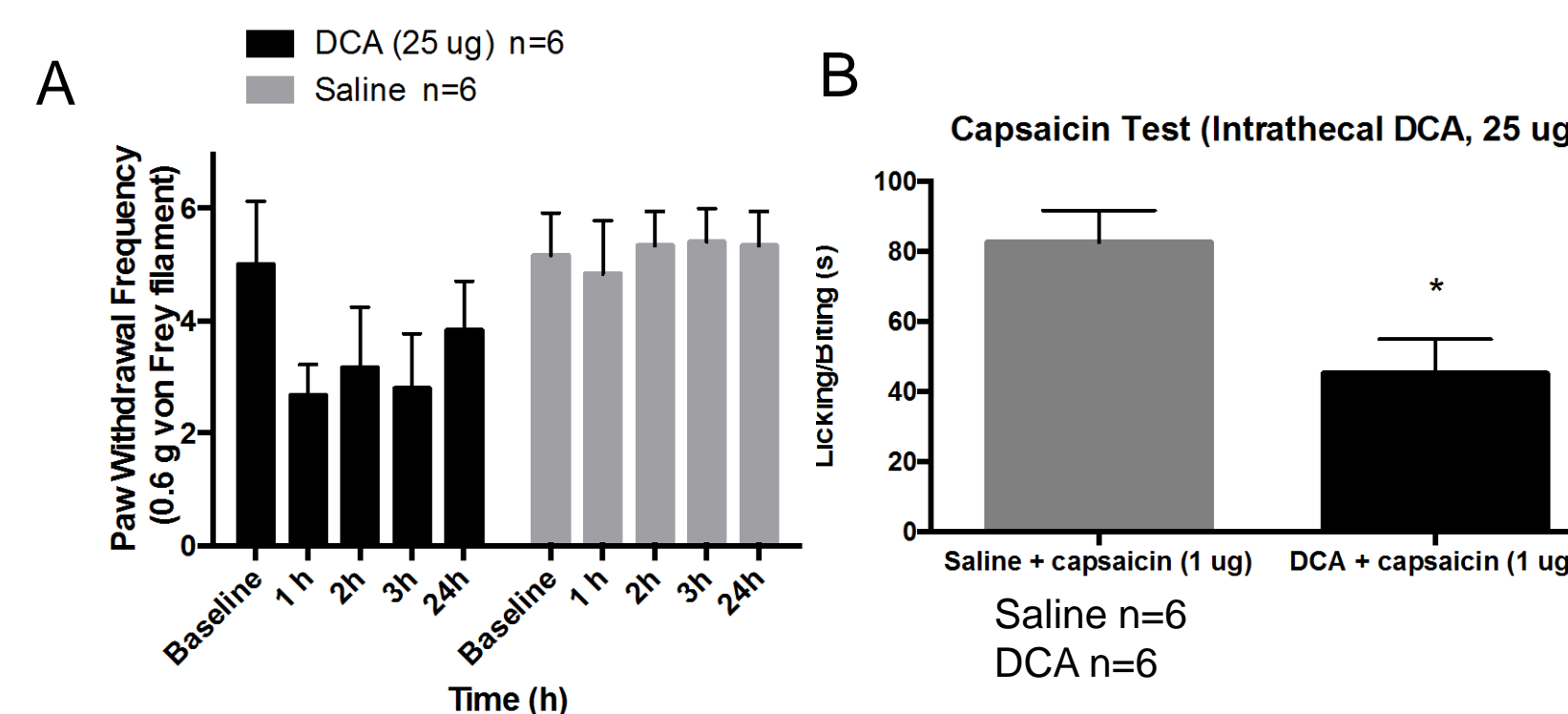


Figure 2. Deoxycholate produces analgesia to acute mechanical and chemical stimuli in naïve CD-1 mice. (A) Intraplantar injection of DCA (25 ug) reduces paw withdrawal frequency to a 0.6g von Frey filament. $p=0.02$ by 2-way ANOVA. (B) Intrathecal injection of DCA (1h pretreatment) attenuates the nocifensive response to capsaicin. * signifies $p < 0.05$ by Student t-test.

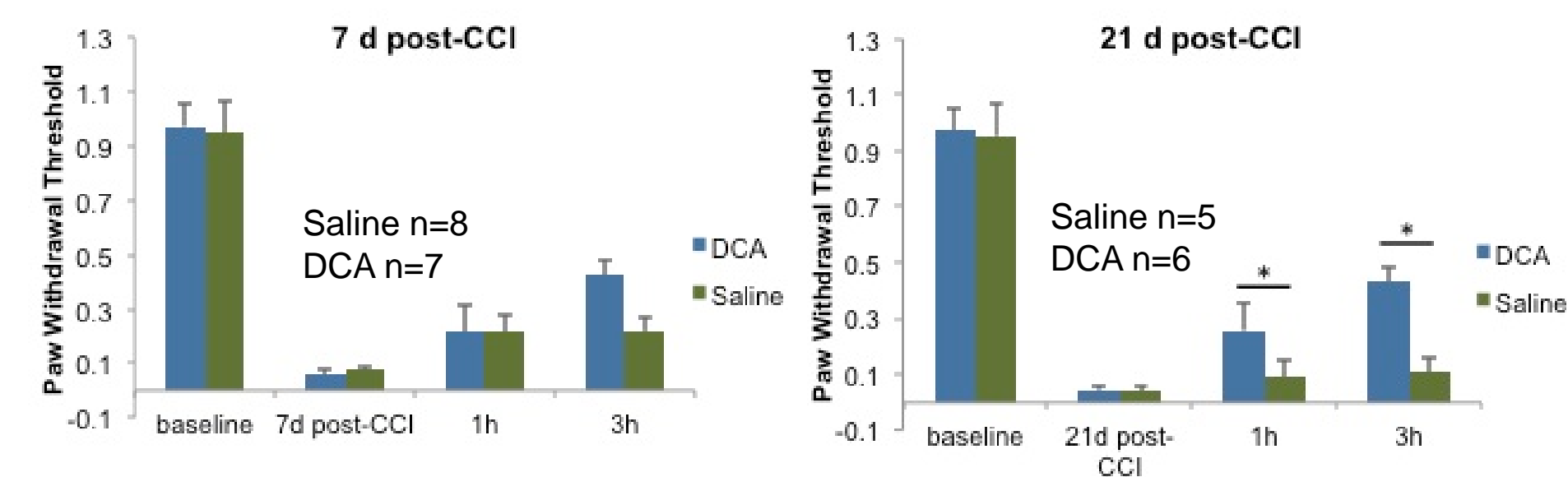


Figure 3. Intrathecal deoxycholate alleviates mechanical allodynia in the chronic constriction injury model at 21 d (late) but not 7 d (early)⁴. * $p < 0.05$ by Student t-test.

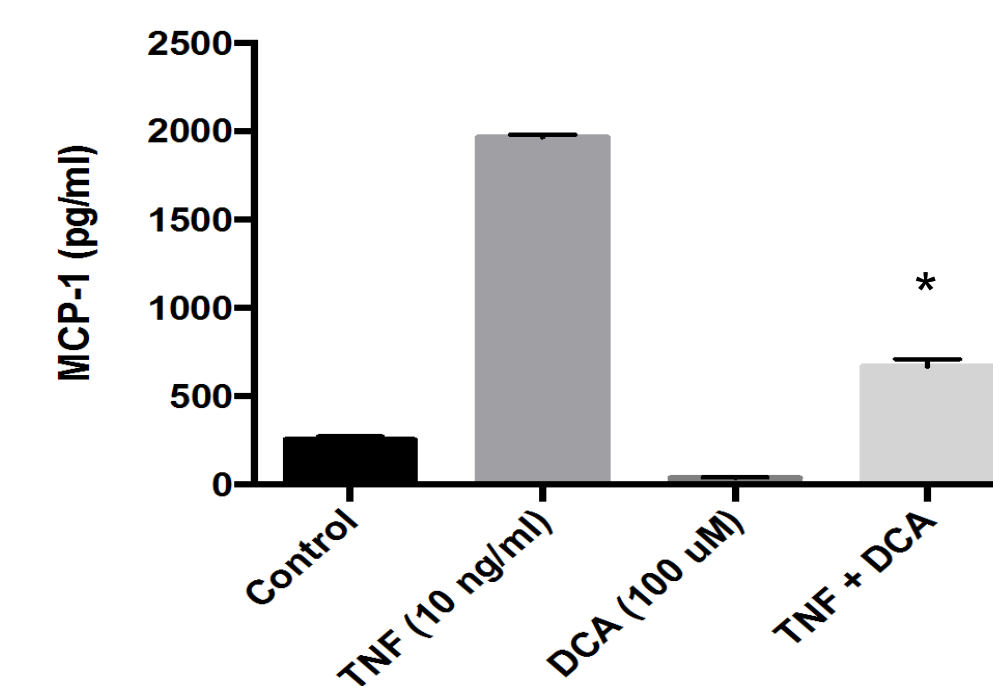


Figure 4. Deoxycholate reduces the release of the chemokine MCP-1 by astrocytes stimulated with TNF *in vitro*. Astrocytes release MCP-1, which contributes to central sensitization and neuropathic pain. Blocking MCP-1 has been shown to attenuate neuropathic pain in mice.³

Conclusions

- Interspecies metabolomics can be used to generate novel, productive leads for pain research
- The bile acid pathway is differentially regulated in pain and non-pain states in mice and humans
- Acute administration of deoxycholate produces analgesia to acute nociceptive stimuli as well as in a chronic, neuropathic pain model
- One part of the overall the mechanism of deoxycholate-mediated analgesia in the chronic setting may be the inhibition of astrocyte contribution to the central sensitization and neuropathic pain.

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4. Behavioral testing performed by Sarah Taves, PhD



Inflammatory Biomarkers in Patients with Persistent Post-operative Pain after Amputation

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Abstract:

- Persistent post-operative pain is one of the most feared outcomes in perioperative medicine.
- Patients undergoing amputation have a greater than 40% incidence of persistent pain after surgery
- 10% of those have pain that significantly alters functional status.
- The question of how acute post-surgical pain becomes chronic after amputation remains unresolved.
- In a post-amputation population, we explored the changes in plasma cytokine concentration that reflect systemic inflammatory state.

Methods:

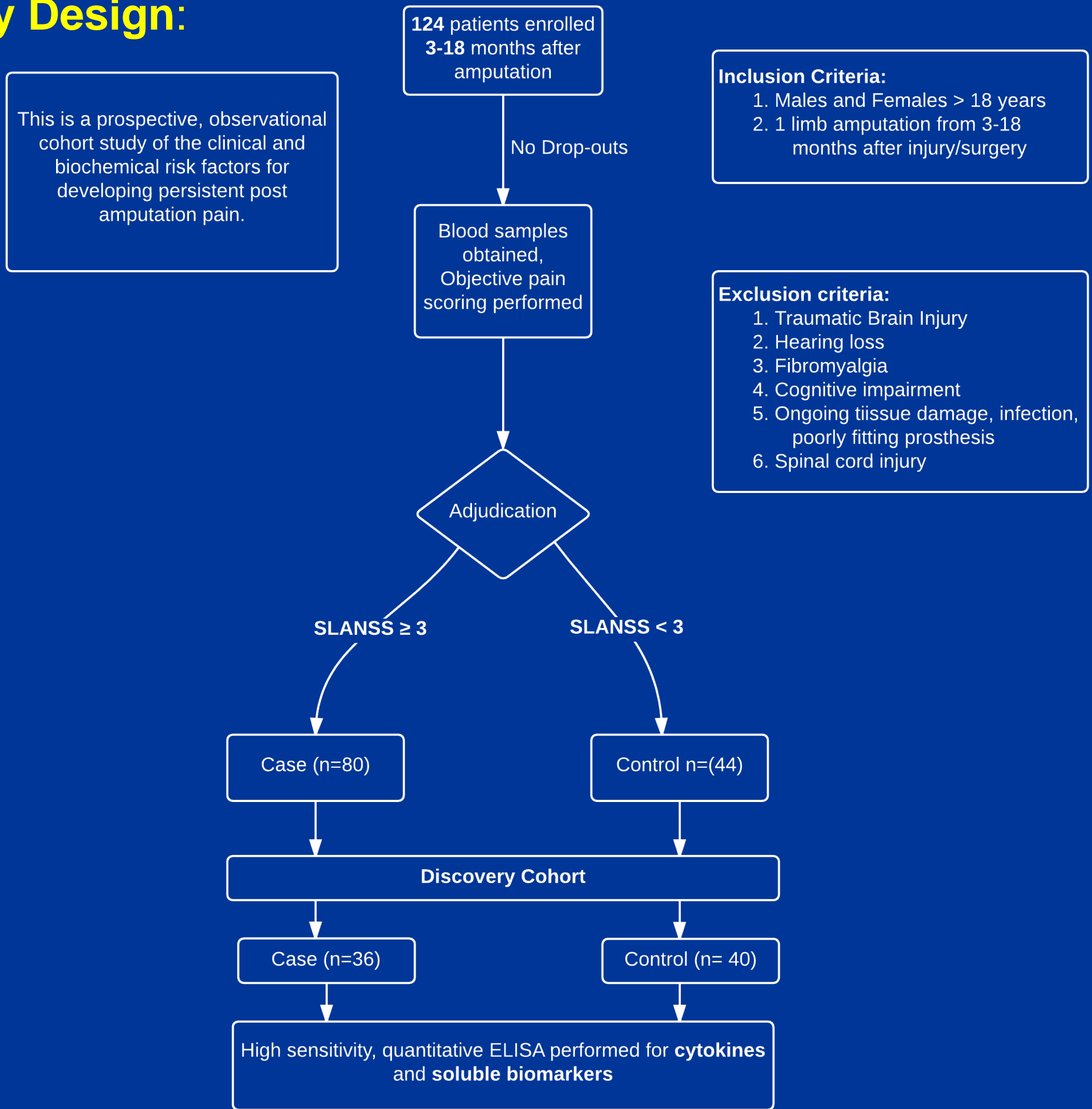
- We used plasma samples from a prospective, cohort study that enrolled patients 3-18 months after amputation.
- Patients underwent objective pain testing, examination and blood sampling at the time of enrollment.

Demographics Table	Control (N=40)	Case (N=36)	p-value
	Mean (SD)	Mean (SD)	
Age	25.20	27.69	0.1526*
Body Mass Index	25.66	26.78	0.1427*
Time since amputation	7.55	8.94	0.2243*
Smoking (ppd)	0.63	0.60	0.8168*
	N (%)	N(%)	
Male	40 (100)	35(97)	0.9577^
Smokers	25 (63)	21 (58)	0.8918^
Ethnicity	N (%)	N(%)	
American Indian/Alaska Native	0 (0)	0 (0)	1.0000^
Asian	2 (5)	1 (3)	1.0000^
Native Hawaiian or Other Pacific Islander	0 (0)	0 (0)	1.0000^
Black or African American	3 (7)	3 (8)	1.0000^
White	37 (93)	32 (89)	0.8836^

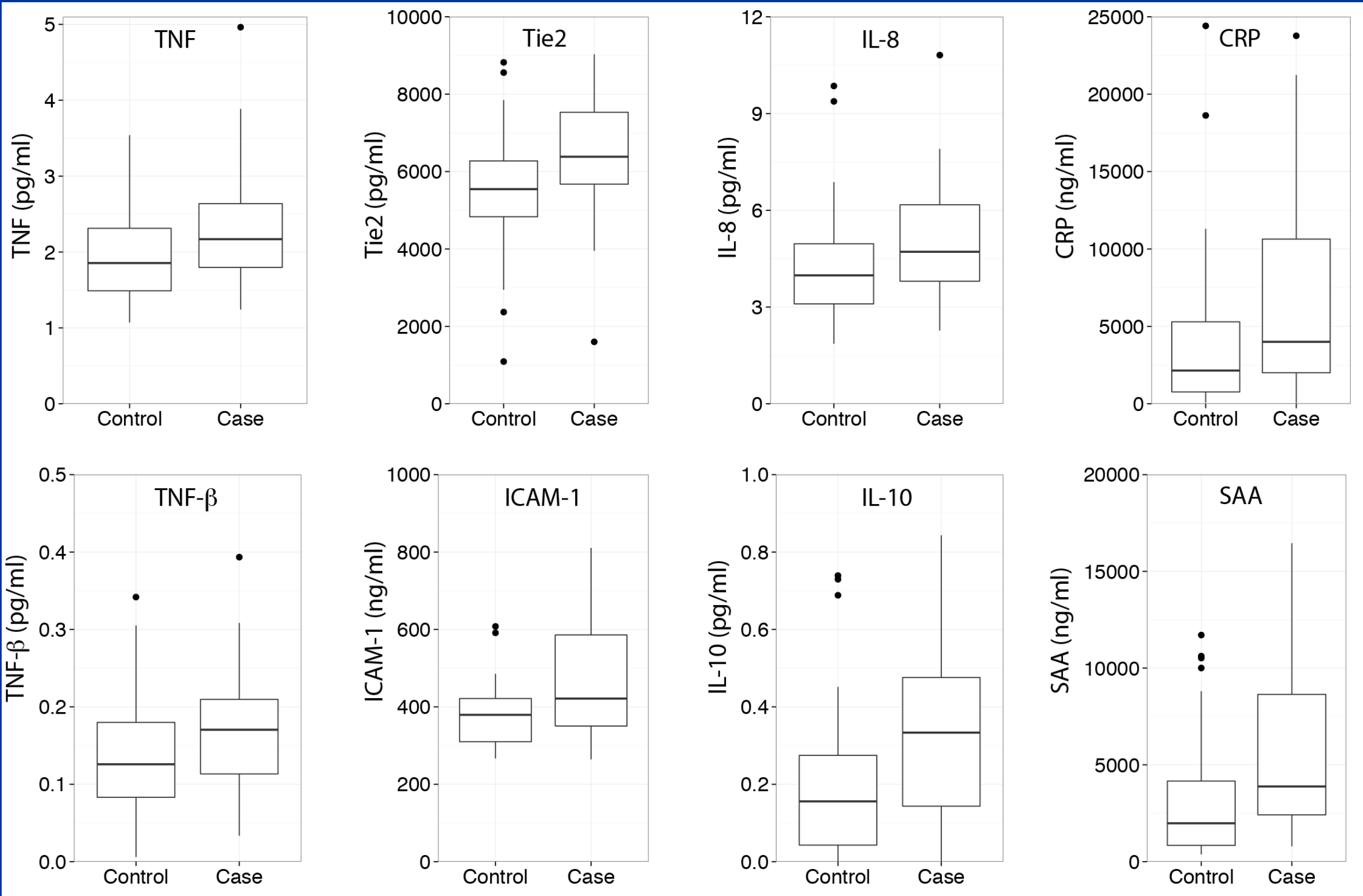
*p-value generated from a two-tailed t-test test. ^ p-value generated from chi-squared test

- Patients with a SLANSS pain severity score of ≥ 2 were categorized as cases and patients with a pain severity score of < 2 were considered controls by a physician adjudication panel.
- Plasma samples from the VIPER (Veterans Integrated Pain Experience Research) study were analyzed with a high-sensitivity enzyme-linked immunoassay (ELISA) for 37 soluble biomarkers including chemokines and cytokines that are involved in neuroinflammation.

Study Design:



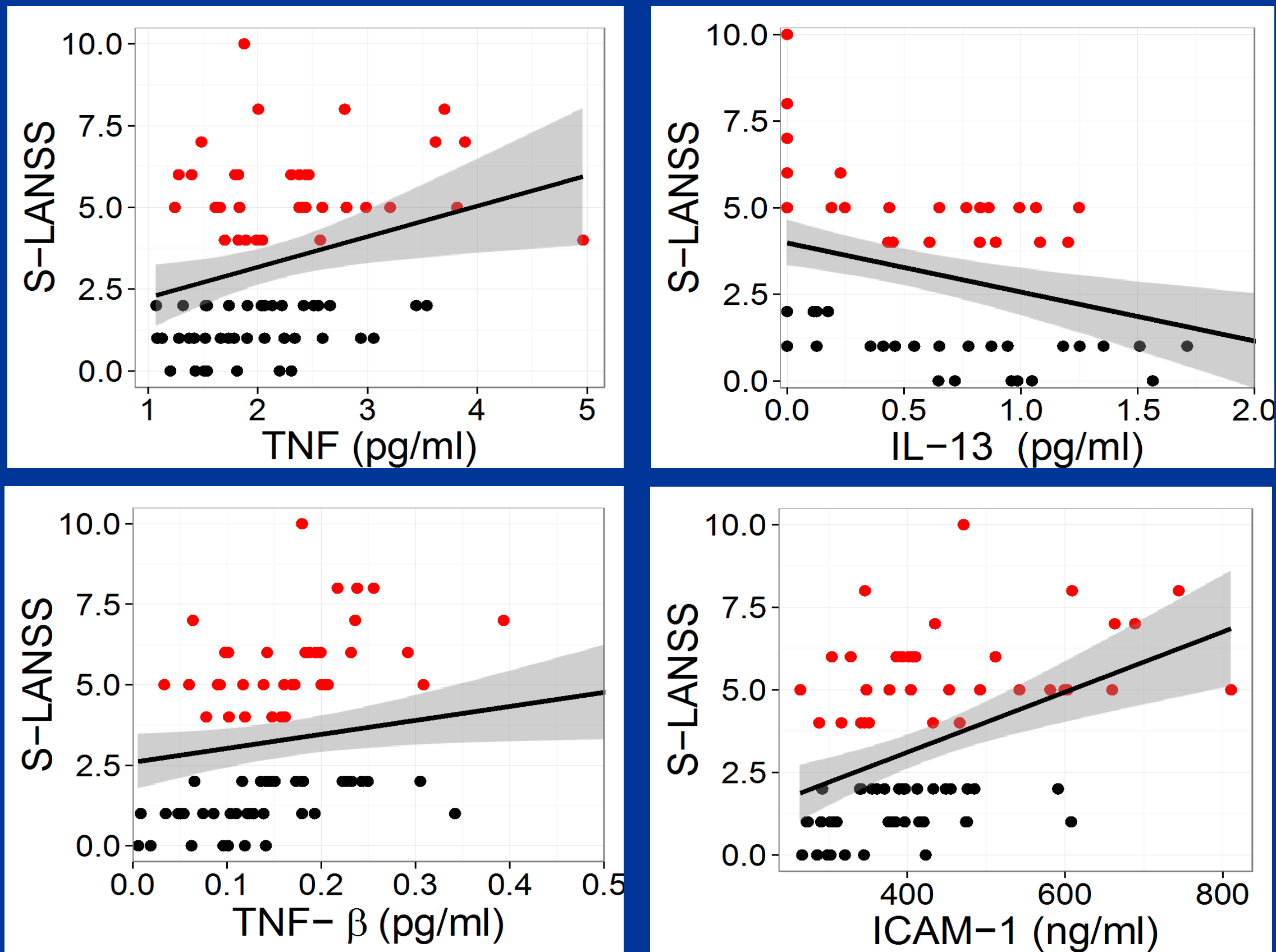
Results: Case subjects exhibited higher serum levels of the pro-inflammatory mediators TNF, IL-8, TNF- β , CRP, SAA, and Tie-2, and the anti-inflammatory mediator IL-10, compared to control group values.



Although these differences were statistically significant individually ($p < 0.05$), they did not withstand multiple comparison correction.

Results:

- There were several correlations between SLANSS severity score and Visual Analog Scale (VAS) score that remained significant even after adjustment for multiple comparisons.
- TNF-b and ICAM-1 were positively correlated with SLANSS score, while IL-13 exhibited a negative correlation.
- TNF and TNF-b were both positively correlated with VAS score.



Conclusion:

- Systemic inflammation after amputation is potentially a driver for the transition of acute pain to chronic pain. We observed an overall increase in inflammatory cytokines in cases versus control, suggesting that systemic inflammation has a role in the development and maintenance of persistent pain after amputation.
- We observe a negative correlation between SLANSS severity score, an indicator of neuropathic pain, and IL-13 indicating that anti-inflammatory cytokines may be protective in the development of chronic pain after amputation.
- This data has led us to hypothesize that pro-inflammatory cytokines drive persistent pain while anti-inflammatory cytokines, such as IL-13, serve a protective role. We additionally found elevations of ICAM-1 in cases relative to controls and a positive correlation with S-LANSS severity score, suggesting that leukocyte trafficking may enhance neuropathic pain phenotype.
- These results, while interesting, need follow-up in a larger scale, prospective study and validation in animal models of persistent pain before these biomarkers are seen as heralding chronic pain after surgery.



Regional Anesthesia Catheters Reduce the Incidence of Neuropathic Post-Amputation Pain: Results from the VIPER Cohort of Injured Military Personnel

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Background

- Chronic pain is a common problem in injured military service members undergoing amputation.¹
- Most studies of post-amputation pain only discriminate phantom and residual limb pain.²
- Sub-classification of pain phenotypes is an important step in the development of disease-specific therapies.
- An ongoing collaborative study (Veterans Integrated Pain Evaluation Research (VIPER)) between Duke University, Walter Reed National Military Medical Center (WRNMMC) and the Durham VAMC is being conducted to further define post-amputation clinical phenotypes and discover circulating biomarkers of persistent pain.
- Here we make a report on 124 military service members who have undergone clinical assessment and phenotypic adjudication.

Methods

Phenotypic Assignment

After IRB approval, the VIPER clinical cohort was assessed using validated questionnaire instruments:

- Brief Pain Inventory (BPI)
- Self-Reported Leeds Assessment of Neuropathic Symptoms and Signs Pain Scale (S-LANSS)
- Complex Regional Pain Syndrome (Budapest Clinical Criteria)
- Phantom and residual limb pain questionnaires
- A formal endpoint adjudication was performed using the algorithm previously reported by our group³
 - Phantom and residual limb pain were discriminated.
 - Residual limb pain was then sub-categorized into a) Neuroma b) CRPS c) Mosaic Neuralgia or d) Somatic.

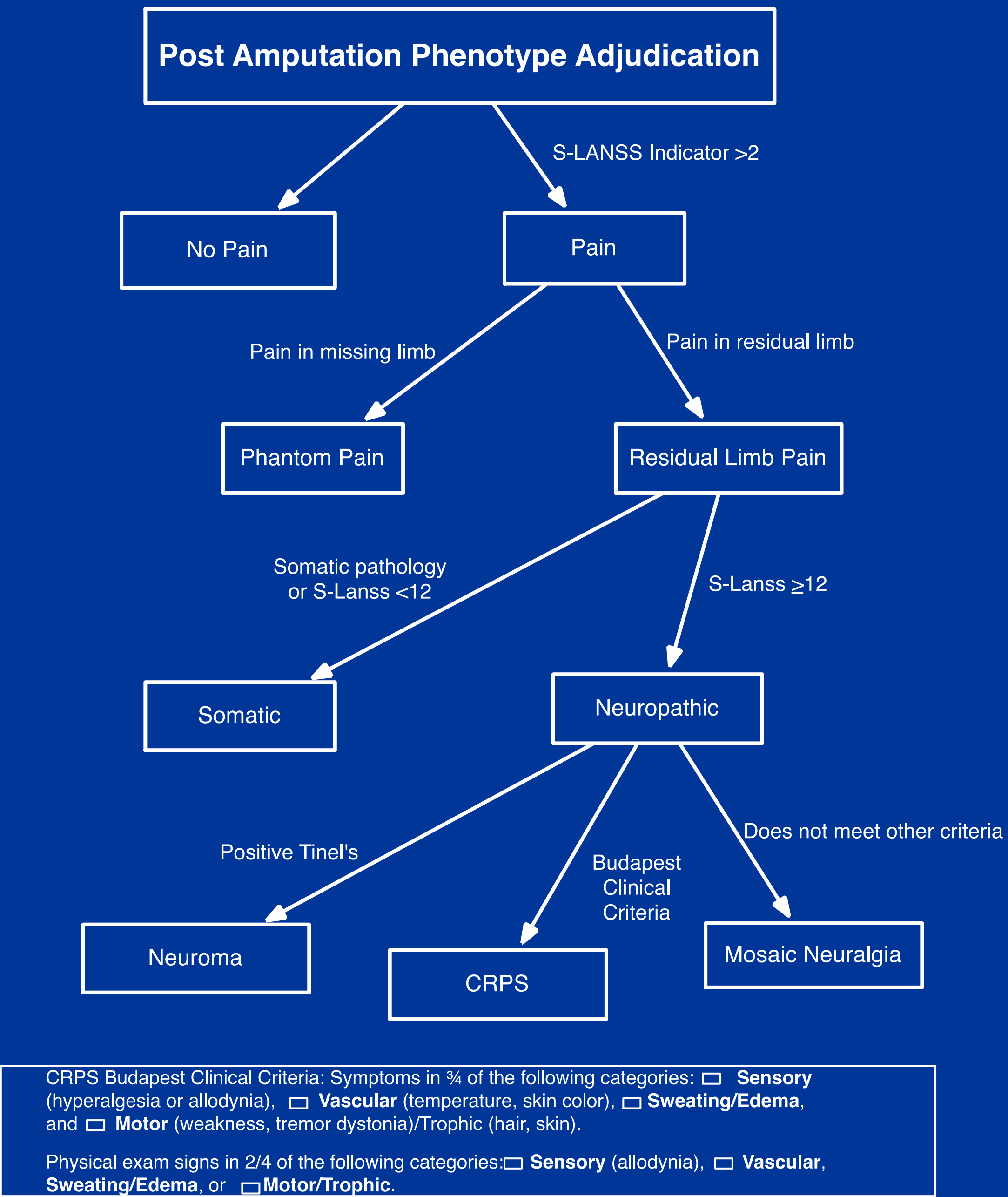


Figure 1: Adjudication algorithm utilized to assign patients to their respective groups.

Results

- Using the Duke Post-Amputation Pain Algorithm (Duke PAPA), we discriminated between several post-amputation pain subtypes in military service members.
 - We found an overall incidence of post-amputation pain (PAP) of 64.5%.
 - When these PAP cases were further sub-categorized:
 - 90% described phantom pain
 - 95% described residual limb pain (RLP)
 - There was significant overlap with these diagnoses, but they did not always co-exist
- Furthermore, of those subjects with RLP the following diagnostic categories were noted:
 - 46.3% neuroma
 - 18.8% CRPS
 - 10% Mosaic neuralgia (neuralgic pain not otherwise specified)
 - 38.8% somatic
- In our analysis of retrospective catheter placement data, we found a significantly decreased incidence of neuropathic pain in patients receiving regional catheters within 7 days of injury.

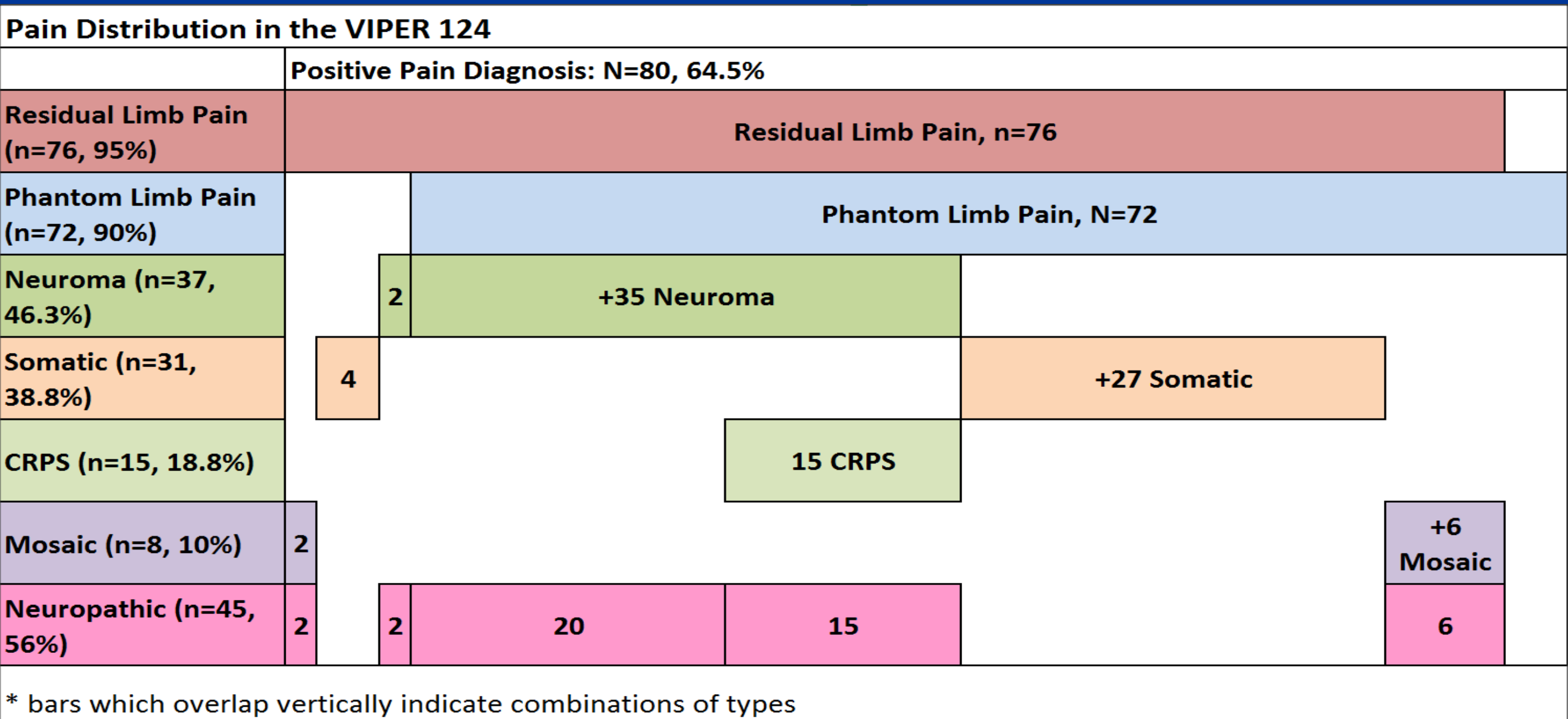


Figure 2: Phenotypic variation in post-amputation pain demonstrating significant overlap between residual limb pain and phantom limb pain. Also, there is substantial overlap between RLP-Neuroma and RLP-CRPS

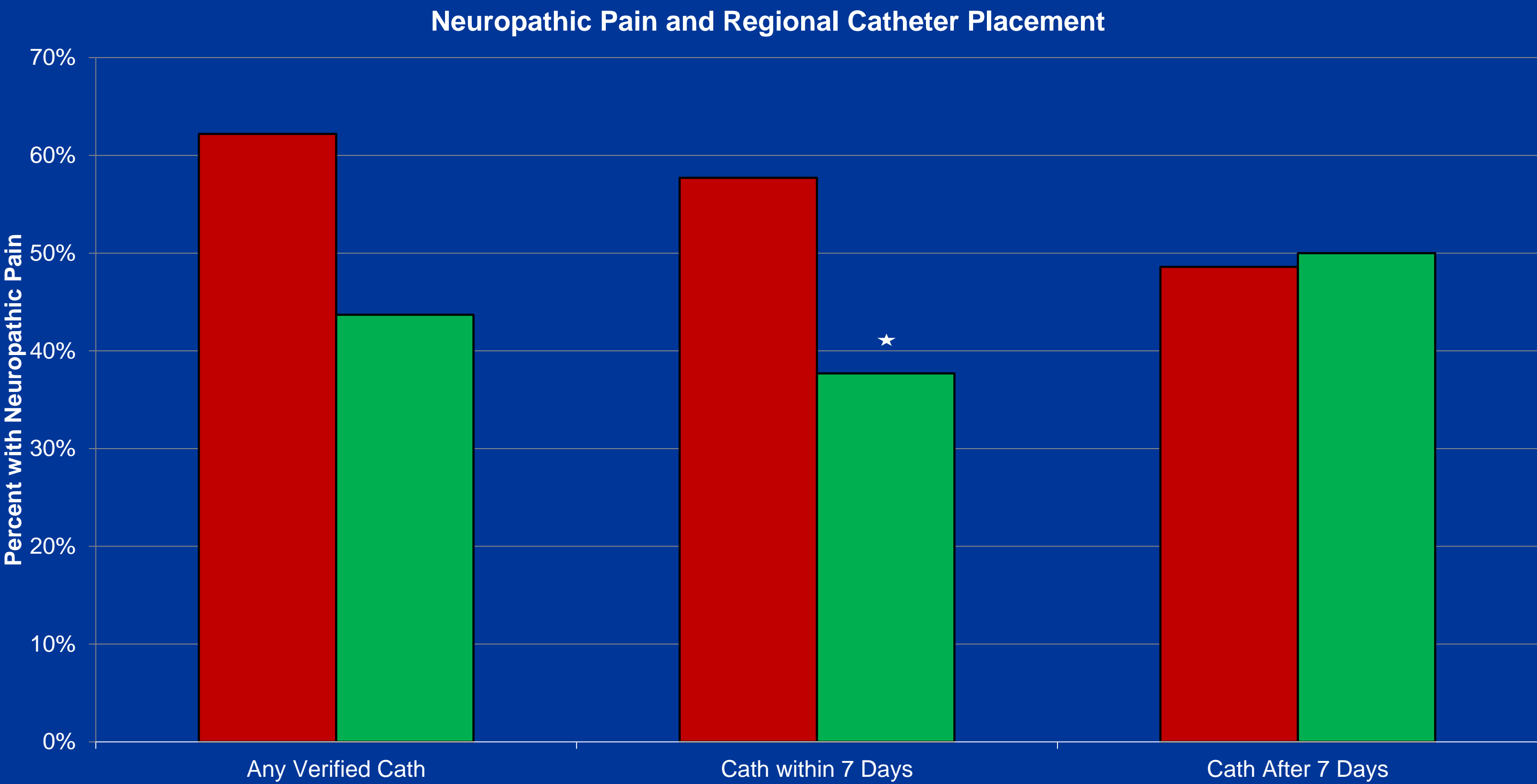


Figure 3: Regional Catheter use and decreased incidence of pain with varying timeframes of placement. The * denotes statistical significance.

Conclusions

- We observed phenotypic complexity of post-amputation pain symptoms in this cohort including:
 - Strong overlap in the diagnoses of phantom and residual limb pain
 - Several distinct subtypes of residual limb neuropathic pain
 - A predominant contribution of neuroma symptoms in service members with residual limb pain
- Additionally, we observed that the use of early regional anesthesia catheters was associated with a decreased incidence of chronic neuropathic pain.

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